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Role of Endothelial Selectins in Wound Repair

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P- and E-selectins are adhesion molecules expressed on activated endothelium and platelets at sites of vascular injury and inflammation. The selectins are important for leukocyte recruitment. Because little is known about the role of selectins in wound healing, we studied cutaneous wound repair of full-thickness excisional skin wounds in mice lacking P-selectin, E-selectin, or both of these selectins. The absence of either selectin alone had no notable effect on healing, and the only deficit observed was a delay in early neutrophil extravasation in the P-selectin-deficient mice. Mice deficient in both P- and E-selectins had markedly reduced recruitment of inflammatory cells and impaired closure of the wounds. Wound sections, studied up to 3 days after wounding, showed significant impairment of neutrophil influx. Macrophage numbers were also reduced in the double mutants at 3 and 7 days after wounding as compared with wild-type mice. Additionally, a wider epithelial gap in the wounds of the P- and E-selectin-double-deficient mice 3 days after wounding indicated delayed keratinocyte migration. These results demonstrate an important combined role for P- and E-selectins in processes leading to wound healing. (Am J Pathol 1997, 150:1701–1709)

P- and E-selectins are adhesion molecules of the selectin family expressed by stimulated endothelium and activated platelets. P-selectin is stored in α-granules of platelets and in Weibel-Palade bodies of endothelial cells.[1–4] P-selectin is rapidly expressed on the endothelial cell surface upon degranulation, and its synthesis can be further increased by cytokines.[5] E-selectin is expressed only after a delay, as it is up-regulated by cytokines and requires *de novo* synthesis.[6] Leukocytes roll on these endothelial selectins, subsequently forming firm adhesions through integrins and migrating across the endothelium to reach the injured or inflamed tissues. By promoting the first step in emigration of inflammatory cells, the selectins play important roles in inflammatory and immune responses.[7–10] P-selectin-deficient mice have shown significant impairment of neutrophil, macrophage, and lymphocyte influx in several acute and chronic inflammatory models[7,11–13]; this impairment is further augmented in mice that are deficient in both P-selectin and E-selectin.[14,15] Although adhesion of leukocytes to E-selectin has been demonstrated *in vitro*,[16] E-selectin-deficient mice have not shown any major phenotype thus far.[17]

Inflammatory cells play a crucial role in wound repair, yet it is not known whether selectins contribute to the wound-healing process by mediating the recruitment of these cells. In the initial inflammatory phase of wound healing, neutrophils and macrophages are recruited. Neutrophils provide defense against invading microorganisms, release oxygen radicals, produce degradative enzymes, and secrete cytokines. Many neutrophils are eventually present in the clot covering the wound and slough off with the eschar as healing progresses.[18,19] Macrophages are critical for proteolysis and removal of debris as well as reconstructive processes.[19–21] They phagocytose organisms, express specific fibronectin variants,[21–23] release cytokines and growth factors including angiogenic factors, and promote the formation of granulation tissue that replaces the provisional matrix.[20,21] Re-epithelialization of the wound takes place by the migration of the keratinocytes from the edges of the wound toward the center

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and overlaps with the inflammatory phase.[illegible] Subsequently, granulation tissue rich in new blood vessels develops and transforms into a scar.[illegible]

Two lines of evidence suggest that leukocytes exert important functions in wound healing. First, depletion of macrophages by corticosteroids and antimacrophage serum delays wound healing,[illegible] although no obvious defects were seen in animals made neutropenic with antiserum.[illegible] Second, delayed wound healing was observed in leukocyte adhesion deficiency type I patients who lack all β_2 integrins and who have defects in several neutrophil functions including neutrophil-endothelial interactions. These defects result in a markedly impaired recruitment of leukocytes, especially neutrophils, into sites of infection in these patients.

As it is well established that selectins expressed on blood vessels are important for recruitment of leukocytes, in the present study we examined the function of the endothelial selectins in wound healing. The approach taken was to analyze cutaneous wound repair in mice lacking P-selectin or E-selectin and in mice deficient in both of these selectins.

Materials and Methods

Mice

Two- to four-month-old 129Sv/C57B1/6 age-matched, wild-type, P-, E-, or P/E- (double-deficient) selectin-deficient female mice generated by gene targeting[illegible] were used in this study. All experimental procedures were approved by the Animal Care and Use Committees of the New England Medical Center and the Center for Blood Research, Boston, MA.

Wounding and Tissue Preparation

Mice were anesthetized with tribromoethanol (0.15 ml/10 g body weight) or methoxyflurane (Metofane, Pitman-Moore, Mundelein, IL), and hair was removed from the back with electric clippers. The skin was cleaned with 70% alcohol, and full-thickness wounds were made by picking up a fold of skin, placing it over dental wax (Polysciences, Warrington, PA), and using a disposable sterile 4-mm punch biopsy (Baker Cummins Dermatologicals, Lakewood, NJ) to punch through the two layers of skin on one flank. In this manner, two wounds were made at the same time on the left side. Later, two wounds were made simultaneously on the right side. For some experiments, wounds were made in mice treated orally with trimethoprim (24 mg/dl) and sulfamethoxazole (120 mg/dl)[illegible] starting 3 days before wounding and continuing for 7 days. The mice were housed in individual cages. At various intervals after wounding, mice were anesthetized, and a transparency was placed over the wound so the wound could be traced. The tracings of the wound opening were photocopied on paper and were cut out and weighed. The weight of the cut-out copy was converted to area by weighing a piece of paper of known area. The mice were sacrificed, and wounds were harvested with 1 to 2 mm of normal skin tissue around them. The wounds were cut in half, fixed in 4% paraformaldehyde (Baxter Diagnostics, Deerfield, IL), and embedded in paraffin, and 7-μm sections were stained with hematoxylin and eosin (H&E). Extravascular neutrophils were counted in the entire section outside the blood vessels using a light microscope (Olympus BX40F at ×40 magnification). The neutrophil infiltrate in the clot/eschar above the wound was very dense; hence, neutrophils could not be counted. Instead, the neutrophils were scored based on their density and the area of the clot/eschar they occupied. In the scoring scheme, 0 indicated absence of a neutrophil band in the clot/eschar and 4 indicated that almost the entire clot/eschar was occupied by neutrophils. The epithelial gap (distance between the leading edge of migrating keratinocytes) was measured in H&E-stained paraffin sections of wounds using a linear grid at ×10 magnification. The area of the granulation tissue in H&E-stained tissues was visualized by a light microscope through a Hitachi CCD camera and was quantitated by an image analysis system (Leica Q-500MC). The paraffin sections were stained for macrophages with F4/80 antibody (American Type Culture Collection, Rockville, MD) modified from the method described by Hume et al.[13] using the biotin-streptavidin system (Zymed Laboratories, San Francisco, CA) and Vectastain ABC kit (Vector Laboratories, Burlingame, CA).

All sections were examined independently by two investigators without knowledge of the genotype.

Myeloperoxidase Assay

Excised wound tissue was washed in phosphate-buffered saline and homogenized in 1 ml of 50 mmol/L potassium phosphate buffer, 0.5% hexadecyltrimethyl ammonium (Sigma Chemical Co., St. Louis, MO), 5 mmol/L EDTA, pH 6.0, at 4°C using a Polytron homogenizer (four bursts of 15 seconds at 11,000 speed). The samples were sonicated for 20 seconds, freeze-thawed three times, and centrifuged at 47,000 × g at 4°C. Supernatants were collected and assayed for myeloperoxidase activity by

adding 0.30 ml of supernatant to 0.970 ml of 50 mmol/L potassium phosphate buffer, pH 5.5, containing 0.2% O-dianosine dihydrochloride (Sigma) and 0.0671% hydrogen peroxide and measuring the change in absorbance at 460 nm over 4 minutes. The assay used was a modification of the method described by Trush et al.[20]

Statistical Analysis

The *P* values were obtained using Student's *t*-test for all statistical analysis except for scoring of neutrophils in clot/eschar, which was analyzed using the Mann-Whitney *U* test.

Results

Neutrophil Recruitment

Neutrophil numbers in the tissue were analyzed 1 hour, 4 hours, 1 day and 3 days after wounding in the selectin-deficient and corresponding wild-type mice. Neutrophils that had migrated outside the blood vessels into tissues 1 and 4 hours after wounding were counted microscopically in H&E-stained sections. In the P-selectin-deficient mice, significantly less neutrophil infiltration was present in the tissues in 1-hour wounds compared with wild-type mice (Figure 1A and Figure 2, A and B); however, by 4 hours, many neutrophils emigrated out of the blood vessels into the wound sections in both the mutant and wild-type mice, and the difference between genotypes was no longer significant (Figure 1A and Figure 2, D and E). E-selectin-deficient mice showed no decrease in neutrophil infiltration at either time point (Figure 1B). In contrast, mice deficient for both P-selectin and E-selectin had markedly impaired neutrophil influx at 1 hour as well as 4 hours (Figure 1C and Figure 2, C and F). By day 1, the large number of neutrophils in the tissues precluded microscopic quantitation. Therefore, myeloperoxidase activity was assayed from the wound sections 1 or 3 days after injury. There was a small increase in myeloperoxidase activity in P-selectin-deficient mice compared with wild-type mice at day 1 (Figure 1D). Again, no difference in the neutrophil influx in the E-selectin-deficient mice was observed (Figure 1E). In contrast, a significant reduction in neutrophil recruitment persisted in mice deficient for both P-selectin and E-selectin in the 1- and 3-day wounds, although the difference between the mutant mice and the wild-type mice narrowed by day 3 (Figure 1F).

We evaluated histological sections 1 day after wounding from both wild-type and P/E-selectin-double-deficient mice. Sections obtained from wild-type mice exhibited a dense infiltrate of inflammatory cells, mainly neutrophils, subjacent to the clot/eschar. In the double-deficient mice, such infiltrates of neutrophils could not be seen in most of the sections (Figure 3, A and B). In a few instances, a small band of neutrophils was observed at the wound edge. By day 3, however, dense neutrophilic infiltrates could also be seen in the P/E-mutant mice, although they were still significantly less prominent than those of wild-type mice (Figure 3, C and D). The density and area occupied by the neutrophils in the clot/eschar visually scored on a scale of 0 to 4 was significantly higher in wild-type mice ($P < 0.0003$; median P/E +/+ 4, P/E −/− 1.75), and the pattern of distribution in the clot/eschar was also different from the P/E-

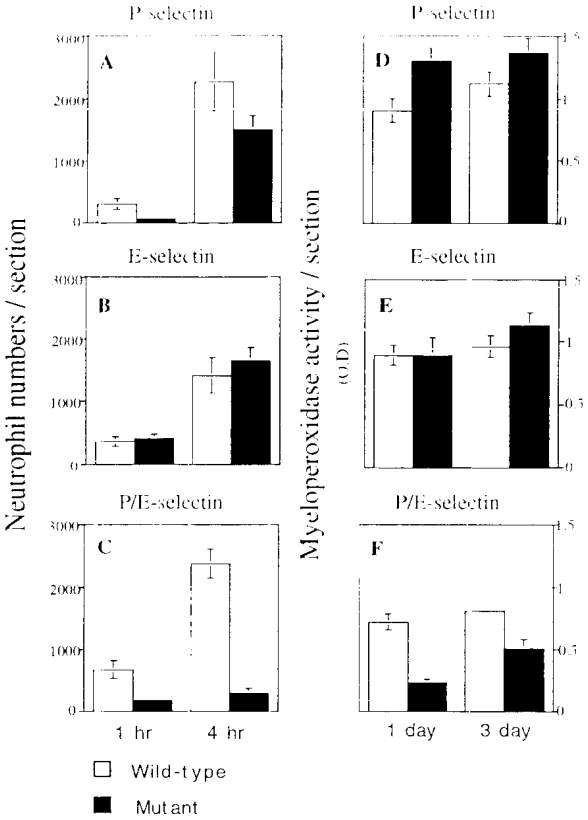

Figure 1. *Neutrophil recruitment in skin excisional wounds.* A to C: *Numbers of neutrophils per section were determined by counting in H&E-stained sections under the microscope.* D and F: *Neutrophil infiltration was established by measuring myeloperoxidase activity in the sections.* A: *Neutrophil numbers were significantly reduced 1 hour after wounding in the P-selectin-deficient mice compared with wild-type mice ($n = 4$ to 5, $P < 0.03$); however, by 4 hours, the neutrophil numbers in the P-selectin-deficient mice increased ($n = 4$, $P = 0.19$).* D: *By day 1, slightly more myeloperoxidase activity was observed in the mutant mice ($n = 11$ to 14, $P < 0.01$).* B and E: *No difference were observed in the E-selectin-deficient mice as compared with wild-type mice.* C and F: *Significantly reduced neutrophil recruitment was observed in the P/E-selectin double-deficient mice compared with wild-type mice at 1 and 4 hours ($n = 8$ to 10, $P < 0.001$), as well as 1 and 3 days ($n = 6$, $P < 0.004$) after wounding. Values are reported as mean ± SEM and n represents the number of tissue sections.*

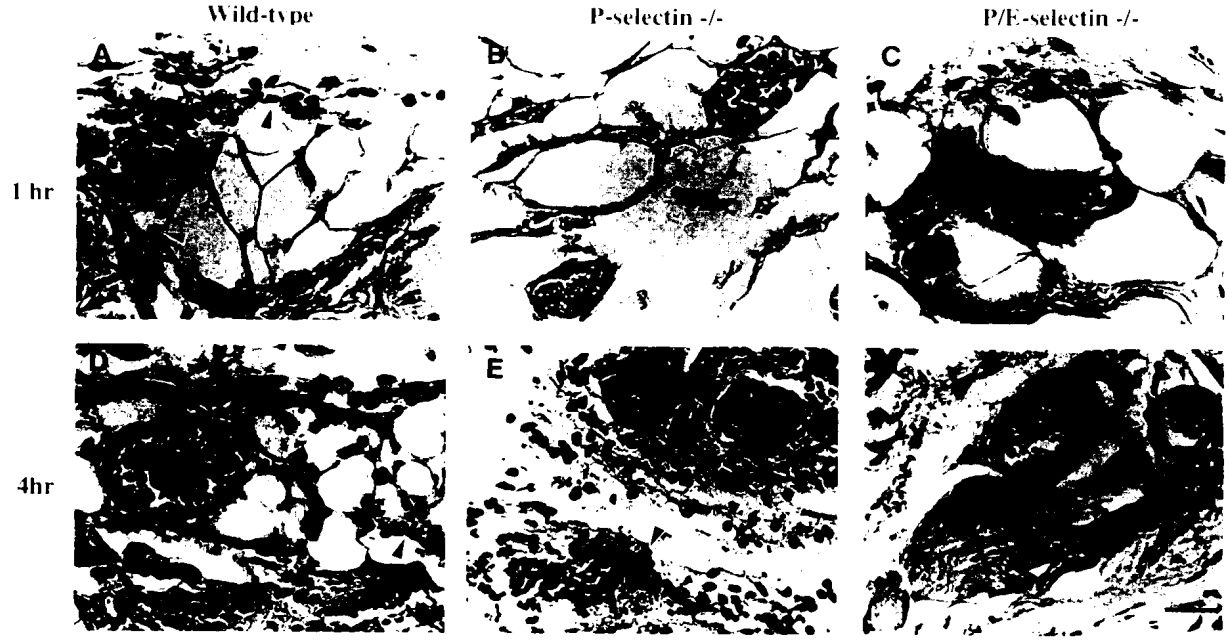

Figure 2. *Histology sections of the wound area after injury. In H&E stained sections of the wounds, neutrophils* (arrow) *were observed extravasating from the blood vessels in the wild-type mice 1 hour after wounding* (A), *whereas few were observed in the E-selectin deficient mice* (B) *and the P/E-selectin double deficient mice* (C). *By 4 hours, neutrophil influx was observed in the P-selectin deficient mice* (E), *however, in the P/E-selectin double deficient mice* (F), *reduced neutrophil recruitment compared with wild-type mice persisted* (D). *Bar, 20 μm.*

selectin-double-deficient mice. The neutrophils in the wild-type mice were observed spreading into the clot/eschar (Figure 3C), whereas in the P/E-mutant mice they were present in a thin band below the eschar (Figure 3D). There was no difference in neutrophil infiltration in the mice deficient for either molecule alone compared with wild-type mice.

Macrophage Recruitment

As macrophages are known to play an important role in wound healing, they were quantitated microscopically after macrophage-specific immunohistochemical staining in the day 3 and day 7 wound sections. Macrophage numbers in the wound tissues of either the P-selectin or E-selectin-deficient mice were similar to those in wild-type animals. However, in mice deficient for both P- and E-selectin, there was a threefold reduction of macrophage infiltration in the wounds at 3 days as well as 7 days after injury compared with wild-type mice (Table 1).

Granulation Tissue

We measured the area occupied by the granulation tissue in sections using an image analyzer. There was no significant difference in the granulation area in day 3 and day 7 wounds in any of the mutant mice compared with their wild-type counterparts (Table 2).

Macroscopic Examination of the Wounds

The wounds were also examined grossly up to 7 days after wounding to assess healing defects. There was no defect in healing on macroscopic examination or in wound area determined from tracings of the wounds in the P-selectin- or E-selectin-deficient mice. However, wound healing appeared to be impaired in the P/E-selectin-double-deficient mice at 3 days as the wound size was larger (Figure 4A).

Keratinocyte Migration

On examination of the tissue sections, we observed that keratinocytes were migrating under the eschar below the dense infiltrate of neutrophils. To evaluate the narrowing of the epithelial gap, the distance between the migrating edges of keratinocytes was measured microscopically at 3 and 7 days after wounding. There was no significant difference in the epithelial gap in wounds of either the P-selectin-deficient or the E-selectin-deficient mice compared with wild-type mice (Figure 4B). In mice that were deficient for both P- and E-selectins, the gap was significantly wider compared with the wild-type mice at 3 days, indicating a defect in migration of the epithelial cells in the mutant mice (Figure 4B). However, by 7 days, the epithelial cells in P/E-selectin-double-deficient mice had advanced similarly to

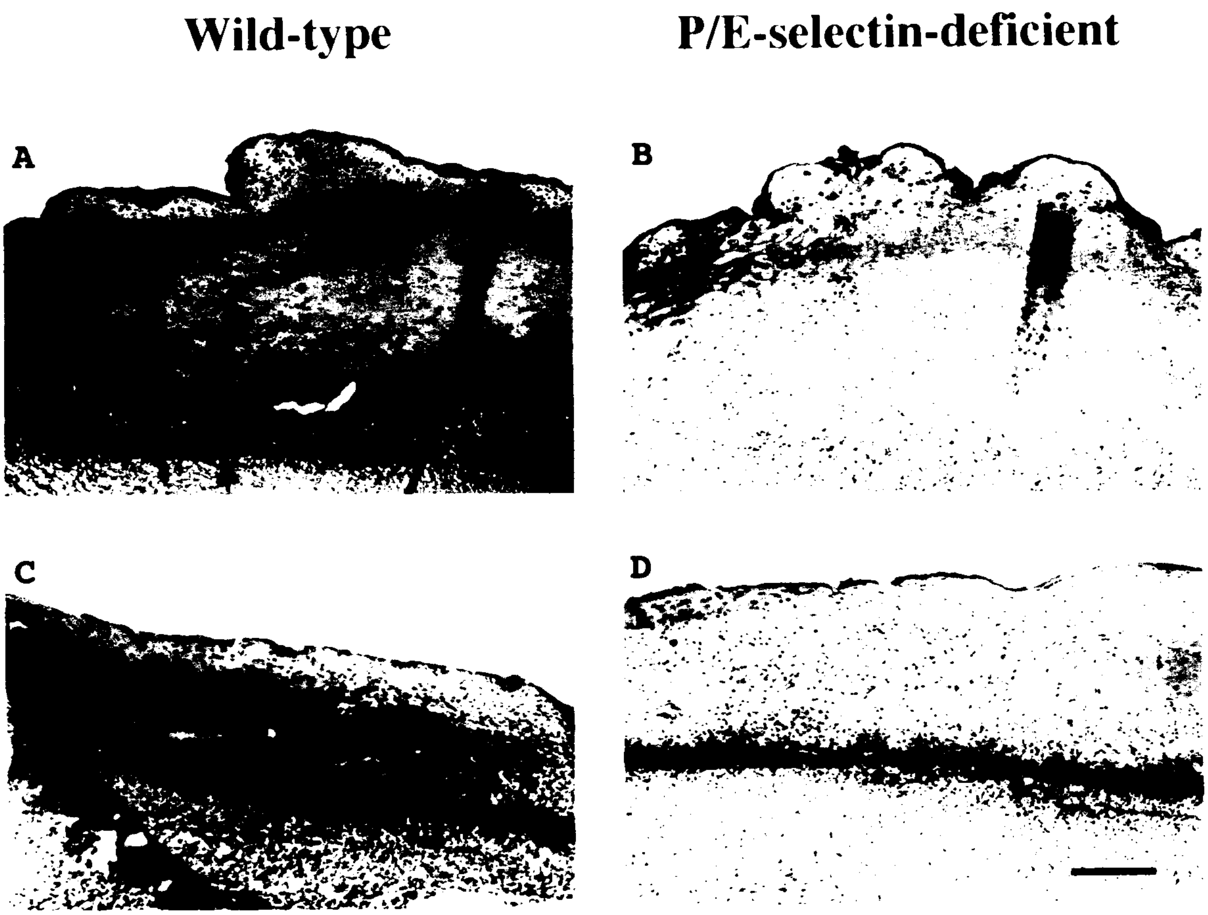

Figure 3. *Histology sections of 1- and 3-day wounds.* A: *In H&E stained sections, dense neutrophil infiltrate* (arrow) *was seen in the clot eschar on top of the wound in wild type mice 1 day after wounds were made.* B: *The P/E selectin double deficient mice were missing the neutrophil infiltrates in 1 day wounds.* C: *At 3 days after wounding in the wild type mice, the neutrophils were seen spreading in the clot eschar.* D: *A less prominent band of neutrophils was now observed in the P/E double deficient mice. Bar, 100 μm.*

those in the wild-type mice, and the epithelial gaps were the same size. The P/E-selectin double-deficient mice have previously been shown to be susceptible to skin infections.[14,15] To confirm that the delay in epithelial migration was not due to infections, wounds were made in mice prophylactically treated with antibiotic regimens known to prevent spontaneous skin infections in these mice. The delay in keratinocyte migration at 3 days persisted in the antibiotic-treated double-deficient mice (P/E +/+ 1.65 mm, n = 5; P/E −/− 2.45 mm, n = 11; $P < 0.01$).

Discussion

Previously, many laboratories including our own have demonstrated an important role for endothelial

Table 1. *Macrophage Recruitment in Skin Wounds*

	P-Selectin			E-selectin			P/E-selectin		
	+/+	−/−	*P*	+/+	−/−	*P*	+/+	−/−	*P*
3 day	30 ± 12 (n = 4)	28 ± 6 (n = 6)	0.8	33 ± 4 (n = 7)	25 ± 5 (n = 6)	0.2	32 ± 4* (n = 14)	12 ± 2* (n = 11)	0.0004
7 day	27 ± 9 (n = 6)	34 ± 6 (n = 6)	0.4	27 ± 5 (n = 8)	30 ± 5 (n = 6)	0.7	18 ± 3† (n = 13)	5 ± 1† (n = 12)	0.0002

[illegible] SEM [illegible]
*P = 0.0004.
†P = 0.0002.

Table 2.

	P-Selectin			E-selectin			P/E-selectin		
	+	−	P	+	−	P	+	−	P
3 days	0.63 ± 0.05 (n = 17)	0.71 ± 0.07 (n = 10)	0.2	0.91 ± 0.14 (n = 8)	0.85 ± 0.1 (n = 15)	0.7	1.27 ± 0.24 (n = 7)	1.04 ± 0.18 (n = 13)	0.4
7 days	1.44 ± 0.16 (n = 17)	1.30 ± 0.13 (n = 12)	0.7	1.13 ± 0.17 (n = 12)	1.20 ± 0.32 (n = 16)	0.3	2.25 ± 0.21 (n = 13)	1.82 ± 0.15 (n = 15)	0.1

Values are expressed as means ± SEM.

selectins in leukocyte homeostasis and emigration of leukocytes to tissues under inflammatory conditions.[1,2,4,5,10,11] It is apparent from several studies that adhesion molecules affect wound repair. Leukocyte recruitment as well as wound healing is defective in patients who lack β2 integrins.[12] Integrins are also involved in the migration and adhesion of keratinocytes, where the members of the integrin family are increased and redistributed to the advancing epidermal edge.[13] Up-regulation of E-selectin has been observed in the capillaries in chronic ulcers of venous insufficiency although its significance is not clear.[14] However, to our knowledge, no studies have evaluated the role of selectins in wound healing.

We studied the role of P-selectin and E-selectin in wound healing by using mice deficient in each of these selectins and in mice deficient in both selectins. In our study, the P/E-selectin-double-deficient mice showed reduced recruitment of neutrophils and macrophages. An overall reduction of neutrophils in wound sections studied up to 5 days after wounding was observed. In addition, delayed recruitment and reduced numbers were also observed in the clot/eschar 1 and 3 days after wounding. Larger numbers of neutrophils were present throughout the clot/eschar in intact mice, whereas the P/E-double-deficient mutants displayed a thin band of neutrophils. It is not clear at this time whether the difference in neutrophil distribution in the clot/eschar of the mutants is due to an overall reduction and a delay of their appearance or whether the migration of neutrophils in the clot/provisional matrix was also affected in the P/E-double-mutant mice. Perhaps P-selectin and integrins expressed on platelets and platelet particles deposited in the clot/provisional matrix may provide a substrate for migration and recruitment.[15–24] In addition, macrophage recruitment was also significantly impaired in the P/E-double-deficient mice up to 7 days after wounding. No major defects in leukocyte recruitment were observed in the mice deficient for P-selectin or E-selectin alone, except for an early, decrease in neutrophil recruitment in the P-selectin-deficient mice. The P-selectin-deficient mice, in fact, had increased recruitment 1 day after wounding. This has also been observed in another inflammatory model of lung vascular-induced permeability where the P-selectin-mutant mice showed increased neutrophil accumulation in the peritoneal cavity at 24 hours ($P = 0.057$).[25] One explanation for this could be a defect in the clearance mechanism of the neutrophils once they are in the tissues.

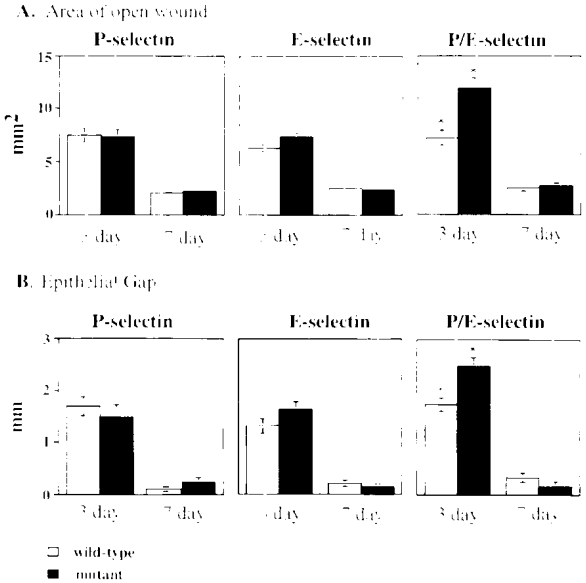

Figure 4. *Wound closure.* A: *Punch-cut, full-thickness wound area was measured in P-selectin–deficient mice compared with wild-type mice 1 day after wounding (n = 11 to 15, $P = 0.07$). The difference was no longer observed 3 days after wounding. No differences were observed in the P-selectin– and E-selectin–deficient mice.* B: *Epithelial gap measured microscopically in H&E stained sections was also reduced in P/E-double-deficient mice 3 days after wounding (n = 15 to 17, $P = 0.009$). Values are expressed as means ± SEM.*

It appears that the absence of either of P-selectin or E-selectin alone does not have a measurable effect on neutrophil recruitment beyond the first hour of cutaneous wounding. The severe prolonged defect in recruitment observed in the double-deficient mice supports the notion that P-selectin and E-selectin work cooperatively to recruit leukocytes. Mice with this combined deficiency have previously been shown to present a much more severe phenotype compared with singly deficient mice demonstrating

leukocytosis,[14,15] hematopoietic alterations,[16] and opportunistic bacterial infections.[14,15]

Although the main defect in the double-deficient mice was the reduced recruitment of neutrophils and macrophages to the wounds, a modest but significant delay in early migration of keratinocytes was also observed. A provisional matrix composed of fibrin, fibronectin, and other plasma proteins is a prominent component of the wound bed at this time. Thus, it is possible that reduced proteolytic degradation and phagocytosis of this provisional matrix may retard the movement of keratinocytes through the provisional matrix.[17] Marked decreases have been observed in epithelial migration in cutaneous skin wounds of plasminogen-deficient mice. In these animals it appears that due to defects in matrix degradation epithelial cells were not able to migrate under the eschar.[18] Keratinocytes in the wound have been thought to express regulators of plasminogen activation. However, many cell types, such as neutrophils, macrophages, and endothelial cells, possess by share this function. These cells may also secrete plasminogen activators and other proteolytic enzymes providing a pericellular environment for degradation of fibrin and other extracellular matrix proteins.[19–21] The effects of fibrin or its breakdown products could also be indirect, regulating keratinocyte migration on other matrix proteins. Additionally, macrophages may provide a plasmin-independent method of cell clearance through phagocytosis and degradation of fibrin.[22] Perhaps in our study, reduced recruitment of inflammatory cells may account for the delayed migration of the keratinocytes by retarding matrix degradation. In fact, as keratinocyte migration seems to be just behind the neutrophil band, these neutrophils appear to be in a good position to enhance matrix degradation thereby allowing keratinocytes to dissect their way through the wound matrix. It is also possible that growth factors and/or cytokines released by the neutrophil and macrophages may affect epidermal migration.[23] The accumulation of these cells by day 5 could also account for the fact that there is no defect in wound closure by day 7. Moreover, a possible overlap in function with other cells, such as keratinocytes, which can regulate plasminogen activator[24] and are a source of growth factors,[25] may compensate for the defect. We have not observed any difference in the size of the granulation tissue; however, we do not know whether more subtle alterations in the wound repair occurred.

As the double-deficient mice are susceptible to infections,[14,15] we were interested to know whether the abnormal epithelial closure was related to wound infections. The P/E-selectin-double-deficient mice given antibiotics showed the same delay in wound closure as was seen in P/E-double-deficient control mice, indicating that the defect was independent of any infection. The markedly impaired recruitment of inflammatory cells in the skin of these mice in response to injury may be responsible for the development of spontaneous chronic skin infections leading to chronic nonhealing ulcerative dermatitis that is reminiscent of the leukocyte adhesion deficiency type I patients.[14] The P/E-selectin-double-deficient mice may prove useful to study the role of selectins in the defense against invasion by various pathogens.

Acknowledgments

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EXHIBIT

G

Absence of P-Selectin Delays Fatty Streak Formation in Mice

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Abstract

P-selectin is expressed on activated endothelium and platelets where it can bind monocytes, neutrophils, stimulated T cells, and platelets. Because recruitment of these cells is critical for atherosclerotic lesion development, we examined whether P-selectin might play a role in atherosclerosis. We intercrossed P-selectin–deficient mice with mice lacking the low density lipoprotein receptor (LDLR) because these mice readily develop atherosclerotic lesions on diets rich in saturated fat and cholesterol. The atherogenic diet stimulated leukocyte rolling in the mesenteric venules of LDLR-deficient mice, and the increase in adhesiveness of the vessels was P-selectin–dependent. Most likely due to the reduced leukocyte interaction with the vessel wall, P-selectin–deficient mice on diet for 8–20 wk formed significantly smaller fatty streaks in the cusp region of the aortae than did P-selectin–positive mice. This difference was more prominent in males. At 37 wk on diet, the lesions in the LDLR-deficient animals progressed to the fibrous plaque stage and were distributed throughout the entire aorta; their size or distribution was no longer dependent on P-selectin. Our results show that P-selectin–mediated adhesion is an important factor in the development of early atherosclerotic lesions, and that adhesion molecules such as P-selectin are involved in the complex process of atherosclerosis. (*J. Clin. Invest.* 1997. 99:1037–1043.) Key words: cell adhesion • hypercholesterolemia • animal model for atherosclerosis • macrophage • endothelium

Introduction

An early event in atherosclerosis is the attachment of circulating monocytes to injured or otherwise stimulated endothelium (1–3). This step may involve complementary adhesion molecules on the endothelium and the monocytes. The monocytes then migrate across the endothelium and ingest lipids, thus becoming foam cells in fatty streaks. Platelets interacting with the transmigrating monocytes contribute additional cholesterol and growth factors to the lesion (2, 4). An adhesion receptor that can mediate both the binding of monocytes to injured endothelium and of activated platelets to monocytes is P-selectin.

P-selectin is a member of the selectin family of adhesion receptors that mediates interaction among vascular cells through a COOH-type lectin domain located at the NH_2 terminus of the molecule. The lectin domain is followed by an EGF-like domain, several repeats shared with the complement binding proteins, a transmembrane domain, and finally a short cytoplasmic tail (5, 6). P-selectin is stored in Weibel-Palade bodies of endothelial cells (7, 8) and alpha-granules of platelets (9, 10). Upon stimulation by agonists, many of which are generated by injury (11), P-selectin is rapidly exocytosed to the cell surface where it can bind monocytes, neutrophils, platelets, and lymphocytes (5, 12). In addition, the synthesis of P-selectin is upregulated upon stimulation by various cytokines (13, 14). P-selectin is required for efficient recruitment of neutrophils in acute inflammation (15), of macrophages in later stages of the inflammatory response (16), and of $CD4^+$ T cells in contact hypersensitivity response (17). P-selectin, therefore, functions in both acute and chronic processes and could play a role in the development of vascular lesions in atherosclerosis.

The mouse subjected to a high-fat diet has become an accepted animal model to study factors involved in the development of atherosclerosis (18). To address the possible role of P-selectin in the formation of atherosclerotic lesions, we have taken advantage of two genetically altered murine lines generated by homologous recombination in embryonic stem cells. The first line lacks P-selectin (15). In these mice, leukocytes and platelets no longer roll on the endothelium of stimulated blood vessels, indicating that the initial adhesion contact of these cells is disrupted (15, 19, 20). The second line represents an animal model of the human disease, homozygous familial hypercholesterolemia. These mice lack the low density lipoprotein-receptor (LDLR)[1] (21) and develop prominent atherosclerotic lesions when fed a high-fat diet (22). We have intercrossed the P-selectin–deficient mice with the LDLR-deficient line, thus developing an animal model highly susceptible to atherosclerosis in which the proposed role of P-selectin in lesion development could be tested.

Methods

Mice. P-selectin–deficient (P-selectin $^{-/-}$) mice were descendants of F2 intercrosses between the C57BL/6 and 129Sv strains (15). P-selectin $^{-/-}$ mice from this background were intercrossed with LDLR-deficient

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1. *Abbreviation used in this paper:* LDLR, low density lipoprotein receptor.

(LDLR) mice, also from a C57BL/6J and 129Sv hybrid background (21). Littermates from the F2 generation of this intercross were genotyped by Southern blotting and used to establish LDLR P-selectin matings and LDLR P-selectin matings. The progeny of these matings were used in our study. Mice were maintained on 12-h dark and 12-h light cycles and given food and water ad libitum.

Diet. (*a*) A normal chow diet of Agway Prolab 3000 from Agway Inc. (Syracuse, NY) that contained 5.0% (wt/wt) fat was fed to chow control mice in all experiments. (*b*) A commercially available 1.23% cholesterol diet, dairy butter diet for mice from ICN Biomedicals (Aurora, OH) based on the diet described in (23) containing 17.84% (wt/wt) butter, 0.98% (wt/wt) corn oil, and 0.48% (wt/wt) sodium cholate was fed to 6–8-wk old LDLR P-selectin or mice for varying lengths of time as described below.

Cholesterol determination. Blood was obtained from the retroorbital venous plexus of nonfasted mice. Cholesterol concentrations (24) in total plasma and lipoprotein fractions were measured using an enzymatic microtiter assay with the A-Gent Cholesterol Reagent from Abbott Laboratories Diagnostic Division (Chicago, IL). The Matrix Plus Cholesterol reference kit from Verichem Laboratories (Providence, RI) was used for standards, and Lipid Control-N and -E from Sigma Diagnostics (St. Louis, MO) for interassay standardization.

Triglyceride determination. Triglyceride concentrations were determined using a microtiter enzymatic assay and the Triglyceride (INT 10) Reagent from Sigma Diagnostics. Standards and controls were as above.

Lipoproteins determination. Lipoproteins were separated using density gradient ultracentrifugation (25). The separation was carried out using an SW-55Ti rotor from Beckman Instruments Inc. (Fullerton, CA) for 44 h at 40,000 rpm at 15°C in a Beckman XL80 ultracentrifuge.

Intravital microscopy. Mice were fed either normal chow or the high fat cholesterol diet for 2 wk before surgery. The mesentery was prepared (15) and venules 15 to 25 μm in diameter were recorded for 10 min. The flux of rolling leukocytes was quantitated by counting the number of cells passing through a perpendicular plane in 1 min. Five 1-min counts were then averaged to determine rolling leukocytes per minute.

Microscopic quantitation of aortic sinus lesions. Hearts from mice on diet for 2, 4, 8, and 20 wk were processed according to Paigen et al. (26). After blood collection from the retroorbital venous plexus, anesthetized mice were killed by cervical dislocation. The heart and attached aorta were removed and placed in 0.9% saline for 1 h, then fixed in 10% buffered formalin for 4–7 d, infiltrated with gelatin, embedded in OCT obtained from Miles Laboratories Inc. (Elkart, IN) and frozen. Mice on diet for 37 wk were bled from the retroorbital venous plexus and hearts were perfused in situ with 4% paraformaldehyde (wt/vol) for 15 to 20 min. The hearts and aortas were collected, placed in 4% paraformaldehyde for 4 to 6 h, followed by 30% sucrose (wt/vol) for 2 to 3 d. Hearts were then embedded in OCT and frozen. All hearts were sectioned using a cryostat and sections discarded until reaching the junction of the heart muscle and aorta where the valve cusps become visible and the aorta is rounded. Once the area was localized, four consecutive 10 μm sections were collected for each slide. Sectioning continued for ~350 μm (9–10 slides/heart) towards the aortic arch and exiting the valve region. Sections were collected onto gelatin-coated glass slides and odd-numbered slides were stained with oil red-O and hematoxylin, and counterstained with light green. Five sections, each 80 μm apart, were scored for each animal without knowledge of the genotype. The area of the lesion was measured with an ocular micrometer. Values reported represent the mean lesion area from five sections for each animal.

Morphometric quantitation of lesions in entire aorta. After 4% paraformaldehyde perfusion, aortae were collected between the subclavian and iliac branches from mice on diet for 37 wk and prepared along with hearts as described above. However, after 2 to 3 d in 30% sucrose, the aortae were stored in 10% formalin for later analysis. The aortae were rinsed in 70% (vol/vol) ethanol for 30 s, stained with 0.5% (wt/vol) Sudan IV in 35% ethanol/50% acetone for 15 min with continuous shaking, destained in 80% ethanol until background was clear, and then washed briefly with water (22). Aortae were opened longitudinally and mounted on slides using glycerol gelatin from Sigma Chemical Co. For quantitation of Sudan IV stained surface area, mounted aortae were visualized through a JVC TK-1280U color video camera into a Leica Q500MC image analysis program (Leica Inc., Deerfield, IL). Percent area covered by lesion was determined by Sudan IV covered area divided by total aorta area.

Identification of macrophages and smooth muscle cells. For identification of macrophages frozen heart sections (10-μm thick) from mice on atherogenic diet for 8 wk were fixed in cold acetone for 5 min then incubated with avidin blocking solution followed by biotin blocking solution (No.004303; Zymed Labs, Inc., S. San Francisco, CA) for 30 min each. Endogenous peroxidase activity was blocked by incubating slides in a solution of 3% hydrogen peroxidase for 5 min. Slides were then incubated with a biotin-conjugated anti-Mac-1 antibody (No. 01712D, dilution 1:5; Pharmingen, San Diego, CA) for 4 h at room temperature followed by Vectorstain ABC reagent from Vector Laboratories. Antibodies were visualized by chromogenic detection with diaminobenzidine as substrate then counter stained with hematoxylin. Antibody was omitted from control section. Smooth muscle cells were stained with a mouse monoclonal antibody against human α-actin directly coupled to horseradish peroxidase (No. U7033, dilution 1:2; Dako Corp., Carpinteria, CA) as described previously (27). Briefly, cryostat cut sections (10-μm thick) of the aortic sinus of the heart from mice fed an atherogenic diet for 37 wk and perfused with 4% paraformaldehyde solution were used. After blocking endogenous peroxidase activity as described above, slides were incubated with the antibody for 4 h. Visualization of antibody and counter staining of slide were same as described above. Control slides were incubated with diluted normal mouse serum.

Statistical analysis. Data are presented as mean±SEM. Statistical significance was assessed by Student's *t* test.

Results

LDLR-deficient mouse model for atherosclerosis. Mice are generally resistant to atherosclerosis and form only small lesions on a high fat diet. Therefore, several mouse models genetically susceptible to atherosclerosis have been developed (28). In some of these models, such as the apolipoprotein E-deficient mouse, lesions develop spontaneously (29–31); in others, such as the LDLR-deficient mouse (21, 22), lesion formation is induced by a high fat diet, and can thus be tightly controlled. Mice deficient in LDLR have elevated lipoproteins (VLDL, IDL and LDL), and in mice on high fat/cholesterol diets cholesterol-laden macrophages accumulate in fatty streaks (Fig. 1 *B*) in the walls of the entire aortic and proximal coronary vessels (22, 32). Because it has not been previously reported whether the lesion in the LDLR-deficient mice can progress to the fibrous plaque stage (28), we have submitted these mice to 8.5 mo of the high fat cholesterol diet. We observed that at this time the lesions in the LDLR-deficient mice indeed progressed to form fibrous plaques. The lesions had a characteristic fibrous cap, positive for α-actin, indicating the presence of smooth muscle cells (Fig. 1 *D*). Considering this normal pattern of lesion progression and the capacity to control lesion onset, we decided to use the LDLR-deficient genetic background to study the role of P-selectin in atherosclerotic lesion development. For this purpose, we intercrossed P-selectin–deficient mice (15) with the LDLR mice (21) to obtain a line deficient in both genes (LDLR P-selectin).

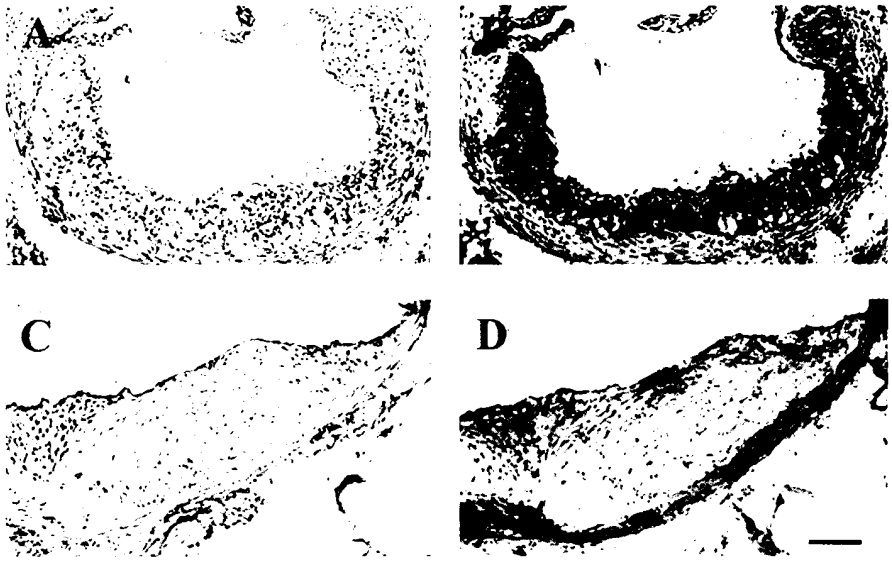

Figure 1. Photomicrographs showing cellular composition of aortic sinus lesions of LDLR-deficient male mice on the high fat cholesterol diet. Sections were obtained from mice that were on the diet for 8 wk (*A*, *B*) and for 8.5 mo (*C*, *D*). (*B*) Section was immunostained with a rat monoclonal antibody to Mac-1 ($\alpha_M\beta_2$), showing a fatty streak laden with Mac-1–positive macrophages. (*D*) Section was stained with a mouse monoclonal anti–α-actin antibody visualizing smooth muscle cells in a fibrous plaque. (*A* and *C*) represent controls without relevant antibody. All sections were counter stained with hematoxylin. Bar, 120 μm.

Diet-induced leukocyte rolling. LDLR mice, with and without P-selectin, were fed either mouse chow or a high-fat/cholesterol diet. To examine the systemic effect the diet may have on leukocyte interaction with blood vessel walls, the mice were subjected to intravital microscopy of mesenteric venules (15) after 2 wk on the diet. The number of rolling leukocytes in the venules was increased threefold in the LDLR /P-selectin mice on the high fat/cholesterol diet in comparison with LDLR /P-selectin mice maintained on mouse chow (Fig. 2). This effect was dependent on P-selectin, as the numbers of rolling leukocytes remained minimal in LDLR /P-selectin mice on either diet (Fig. 2). Our results indicate that the high fat/cholesterol diet led to an increase in leukocyte interaction with the vessel wall of the mesenteric venules. This could be due to either increased endothelial expression of P-selectin or changes in the P-selectin ligand on the leukocytes induced by the atherogenic diet.

Total plasma cholesterol and triglyceride levels. To study the role of P-selectin in atherosclerotic lesion development, we first made sure that the LDLR /P-selectin and LDLR /

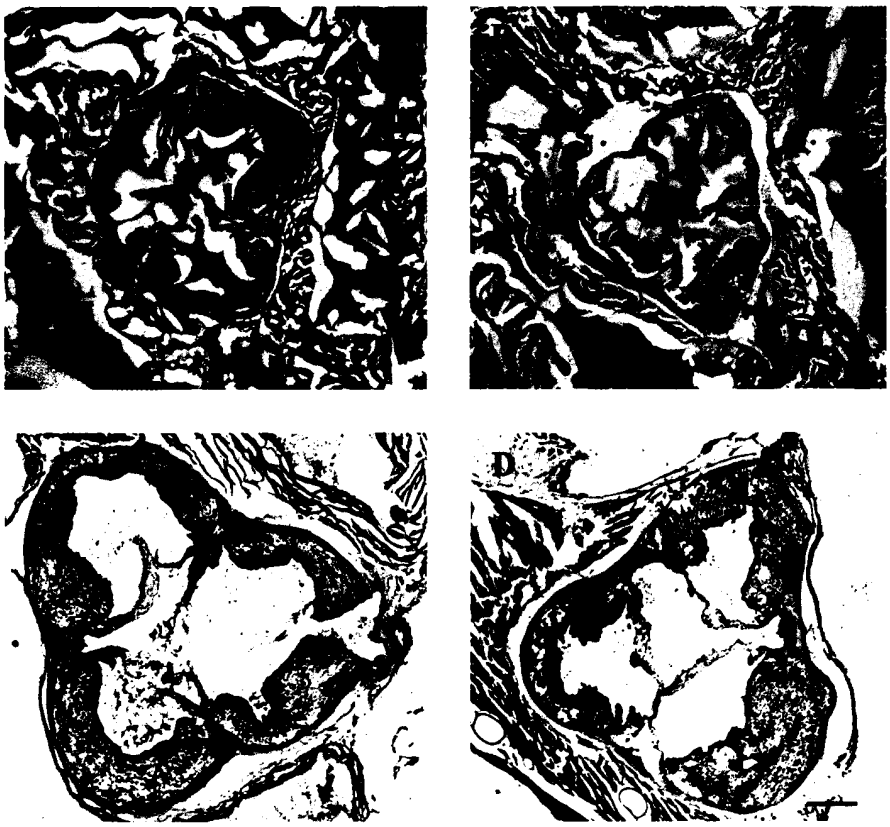

Figure 4. Representative atherosclerotic lesions in LDLR-deficient male mice. Male mice were killed after 8 wk (*A*, *B*) and 37 wk (*C*, *D*) on the high fat cholesterol diet. 8-wk hearts were fixed in 10% buffered formalin. 37-wk hearts were perfused with 4% paraformaldehyde and the processed hearts were stained (26). LDLR /P-selectin (*A*, *C*) and LDLR P-selectin (*B*, *D*) aortae with lesion areas close to the mean value are shown. Arrowheads indicate lesions in the 8-wk samples where the difference in size between the two genotypes is pronounced. With increasing time on diet, the oil red-O–positive staining has progressively changed from a uniform staining pattern in the typical fatty streak (*A*, *B*) to more luminal distribution in the fibrous plaque type lesion (*C*, *D*). This change occurred equally in both P-selectin–positive and –negative hearts. Bar, 300 μm.

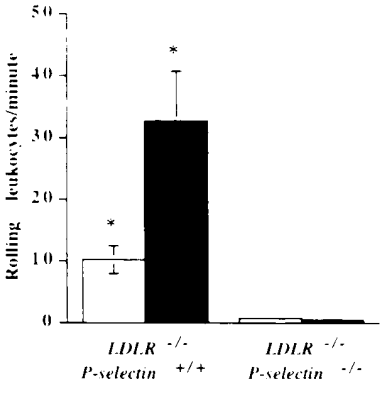

Figure 2. Leukocyte rolling in LDLR-deficient mice on high fat cholesterol diet. LDLR P-selectin and LDLR P-selectin male mice were fed either normal mouse chow (*open bars*) or the high fat cholesterol diet (*black bars*) for 2 wk. Mice were anesthetized, and a midline abdominal incision was made to expose the mesentery. The flux of leukocytes was quantitated by counting the number of cells passing through a perpendicular plane in 1 min. Significant leukocyte rolling was seen only in P-selectin–positive mice, and this was stimulated further by the atherogenic diet. n = 7–11; * comparison by Student's t test P < 0.02.

P-selectin mice responded similarly to the high fat/cholesterol diet as demonstrated by comparable levels of cholesterol and triglycerides in the blood. After 2 wk on the high fat/cholesterol diet, the total plasma cholesterol was > 2,000 mg/dl in male and ~ 1,300 mg/dl in female LDLR mice, independent of P-selectin genotype (Table I). At 4 wk, the cholesterol levels in the males decreased to levels detected in the females and remained similar for the length of the study (Table I). The mice maintained on chow diet had lower cholesterol levels, and again these were similar for both genotypes (LDLR /P-selectin 261.3±15.3 mg/dl; LDLR /P-selectin 223.2±13.5 mg/dl). After 8 wk on diet, 5 LDLR animals of each P-selectin genotype and sex were also analyzed for cholesterol distribution among lipoprotein fractions by density gradient ultracentrifugation. Consistent with earlier studies (22), the increased plasma cholesterol levels were mainly due to an accumulation of β-VLDL (not shown). No statistically significant differences in the cholesterol distribution among lipoproteins for the two P-selectin genotypes were detected. Triglyceride levels were also determined for several animals of each group on the high fat/cholesterol diet and were found to be similar among all sexes and genotypes.

Lesion development in the first 8 wk on diet. LDLR mice on chow diet for 8 wk did not develop atherosclerotic lesions. In contrast, all groups of LDLR mice (males or females, positive or negative for P-selectin) developed small lipid-containing lesions in the cusp region of the aorta in the first 2 wk on the high fat/cholesterol diet. There was little change in lesion size between 2 and 4 wk in LDLR P-selectin and LDLR P-selectin mice (Fig. 3). However, the 8 wk aortae had large intimal collections of cholesterol-laden foam cells resulting in conspicuous projections into the sinus region (Figs. 1 and 4). The lesion areas in females were similar for both P-selectin and P-selectin mice (Fig. 3 *B*) but were smaller than in P-selectin males (P 0.0005). Comparison of lesion areas in the 8-wk-old male samples showed that the P-selectin male mice had lesions twofold larger than the P-selectin males (P 0.0001; Fig. 3 *A*, and Fig. 4, *A* and *B*). The gender difference in P-selectin–dependence was reproducible; the data presented in Fig. 3 were combined from two independent experiments yielding identical results (for the 8 wk time point, experiment 1: males P = 0.031, females P = 0.20; experiment 2: males P = 0.023, females P = 0.73). When mean lesion areas of all (male and female) LDLR P-selectin (n = 27) and LDLR /P-selectin (n = 26) animals were compared, the difference between the two groups was also statistically significant (P = 0.0012, Fig. 3 *C*).

Lesions in mice on long-term diet. To examine the role of P-selectin in lesion progression we maintained a group of LDLR P-selectin and LDLR P-selectin mice on the high fat/cholesterol diet up to 8.5 mo. In mice killed after 20 wk on the diet, a sex difference in lesion size was no longer evident (not shown). A slightly larger lesion size was observed in both sexes in the LDLR /P-selectin animals as compared to LDLR /P-selectin , and this 20% difference was statistically significant when females and males were combined (13 LDLR /P-selectin and 14 LDLR /P-selectin ; P < 0.05; Fig. 5). Sections of the base of the aorta showed that, despite their increased size, the lesions were still predominantly of the fatty streak type without an obvious fibrous cap. At 20 wk, the lesion also involved the root of the coronary artery. This occurred in both P-selectin genotypes (not shown).

At 37 wk of diet, the lesion composition appeared qualitatively different from those seen at the earlier time points (Fig. 4). The oil red-O staining was mainly confined to lumena surface rather than being evenly distributed throughout the lesion as seen in the earlier fatty streak lesions. The lesions contained a necrotic core and were positive for α-actin (Fig. 1) indicating that they had progressed to the fibrous plaque stage. Quantitation of lesion size in the cusp region no longer showed a statistically significant difference between P-selectin–positive and –negative animals (Fig. 5). While at 8 wk on the high fat/cholesterol diet we found no obvious lesions in a segment of the aorta dissected between the subclavian branch and iliac branch in either P-selectin genotype (not shown), lesions were numerous in this segment at 37 wk. To quantify the lesions, 5 LDLR P-selectin and 5 LDLR /P-selectin male aortae were stained with Sudan IV (Fig. 6). The percent surface

Table I. Total Plasma Cholesterol (mg/dl) in LDLR-Deficient Mice on 1.23% Cholesterol Diet

		2 wk	4 wk	8 wk	20 wk	37 wk
P-selectin	males	2381 ± 169 (14)*	1359 ± 70 (10)	1093 ± 83 (13)	1344 ± 84 (7)	1538 ± 75 (5)
P-selectin	males	2151 ± 320 (12)	1433 ± 138 (10)	1104 ± 46 (12)	1229 ± 139 (6)	1285 ± 109 (6)
P-selectin	females	1352 ± 81 (18)*	1312 ± 32 (7)	996 ± 36 (14)	1334 ± 146 (7)	1476 (1)
P-selectin	females	1302 ± 53 (12)	1396 ± 119 (13)	1047 ± 25 (14)	1292 ± 194 (7)	1626 ± 268 (4)

Mice were fed high fat cholesterol diet for 2, 4, 8, 20, or 37 wk at which time plasma was collected and analyzed for total cholesterol. The values represent the mean ± SEM. The values in parentheses represent the number of animals evaluated. Comparison by Student's t test *P < 0.0001; P < 0.02.

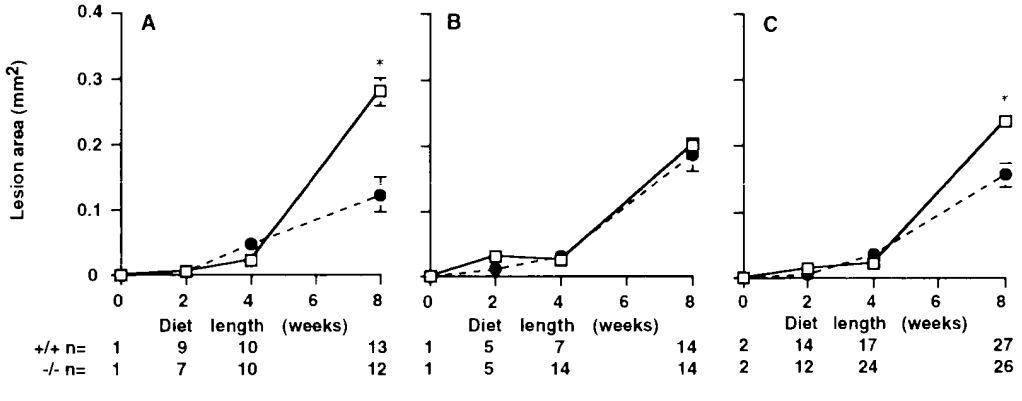

Figure 3. Atherosclerotic lesion size in LDLR-deficient mice with and without P-selectin. (*A*) Male mice only, (*B*) females only, and (*C*) both sexes combined. LDLR P-selectin [+/+] (*open squares*) and LDLR [-/-] P-selectin (*closed circles*) 6–8-wk-old mice were fed the high fat cholesterol diet for 2, 4, and 8 wk. Hearts with ascending aortae were collected, processed, and oil red-O–positive vascular lesions were measured (26). Mean values of five sections/mouse were used for comparison by Student's *t* test. Asterisks indicate statistically significant differences (*A*) $P = 0.0001$, (*C*) $P = 0.0012$. (*B*) The female mice did not show a difference in lesion size.

area occupied by the lesions in these preparations was comparable for both genotypes (LDLR [-/-] /P-selectin [+/+] 53.7% and LDLR [-/-] /P-selectin [-/-] 51.0%, $P = 0.84$).

The above results indicate that P-selectin–mediated adhesion plays an important role in the initial stages of atherosclerotic lesion development, i.e., fatty streak formation, and that its involvement is no longer significant when lesions progress to later stages.

Discussion

The suggestion that Weibel-Palade bodies may be involved in atherosclerosis dates back to the 1970s, as these organelles that are known to contain the adhesion molecules von Willebrand factor (33) and P-selectin (7, 8) were shown to be more numerous at sites of atherosclerotic lesions (34). Increased membrane expression of P-selectin and of two other adhesion molecules for leukocytes, ICAM-1 and VCAM-1, has been shown in human and rabbit atherosclerotic plaques (35–38). Oxidized LDL, a risk factor for atherogenesis, can induce sustained P-selectin expression in human umbilical vein endothelial cells and on rat aortic rings in vitro. Anti–P-selectin antibodies inhibit monocyte adhesion to the oxidized-LDL–treated endothelium (39, 40). Similarly, in an in vivo model using the skinfold chamber, injection of oxidized LDL caused an increase in leukocyte rolling and adhesion to endothelium in both venules and arterioles. Leukocyte adhesion to venular and arteriolar endothelium in this model was significantly reduced by infusion of anti–P-selectin antibodies (41). Comparably, in the LDLR-deficient mice we observed a threefold increase in leukocyte rolling in the mesenteric venules when these mice were subjected to high fat/cholesterol diet for one (not shown) or two weeks (Fig. 2). This increase in adhesiveness was completely prevented by the absence of P-selectin. This indicates that P-selectin is the responsible adhesion molecule and that other adhesion molecules, such as E-selectin, do not play a primary role at this early stage. Because high fat/cholesterol diet induces not only leukocyte rolling in the venules of the LDLR [-/-] mice but also leukocyte transmigration in the aortae leading to fatty streak formation (22, 32), we hypothesized that P-selectin may also be involved in the latter process.

Indeed, we observed that the absence of P-selectin reduces the size of the fatty streaks formed in the LDLR-deficient mice (Figs. 3–5), and this was especially pronounced in the males (Fig. 3 *A*, and Fig. 4, *A* and *B*). It is likely that in these animals there is a sex difference in upregulation of P-selectin, and thus

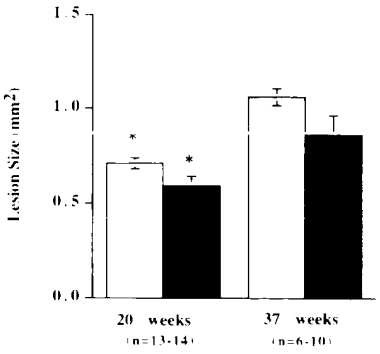

Figure 5. Atherosclerotic lesion size in LDLR-deficient mice with and without P-selectin on long-term atherogenic diet. 6–8-wk-old mice were fed a high fat cholesterol diet for 20 and 37 wk. Hearts were processed and vascular lesions were measured: LDLR P-selectin [+/+] (*open bars*) and LDLR P-selectin [-/-] (*black bars*). Mean values of 5 sections (n = 1 mouse) were used for comparison by Student's *t* test. *$P = 0.05$.

Figure 6. Aortae of LDLR-deficient mice on atherogenic diet for 37 wk. Aortae of male mice were dissected, opened, and stained with Sudan IV. (*A*) shows an LDLR P-selectin [+/+] and (*B*) shows an LDLR P-selectin [-/-] specimen.

it may play a more important role in the male mice than in females. Recently, studies in vitro with human umbilical vein endothelial cells, have shown that the presence of the female sex hormone 17β-estradiol strongly inhibited the IL-1 induced upregulation of the leukocyte adhesion molecules ICAM-1, VCAM-1, and E-selectin (42). Although the effect of this hormone on P-selectin expression, which is also up regulated by cytokines (14), was not examined in that study, we hypothesize that P-selectin may be under a similar regulatory effect of estrogen. Downregulation of P-selectin expression by estrogen may at least in part explain why P-selectin plays a more dominant role in the male mice. Similarly, less expression of several leukocyte adhesion molecules in the LDLR-deficient female mice combined with lower cholesterol levels at 2 wk, may result in the formation of smaller fatty streak lesions in the females than seen in the males ($P = 0.005$; Fig. 3). Others found a comparable sex difference in lesion size in LDLR-deficient mice maintained for 6 mo on a 1% cholesterol diet (32). In addition, the LDLR-deficiency changes the lipoprotein profile of the mouse so that it becomes similar to that of humans, with non-HDL lipoproteins dominating the lipoprotein species (22). Therefore, LDLR-deficiency has made mice more like humans (43) with respect to gender-related susceptibility to diet-induced atherosclerosis. This sex difference combined with our demonstration that the LDLR-deficient mice develop similar fibrous plaque lesions (Fig. 1) as seen in humans, shows that the LDLR-deficient mice indeed constitute an interesting animal model to study the genes involved in atherogenesis.

From our study it is apparent that P-selectin played a significant role in the formation of the fatty streak type lesions (8–20 wk on diet) and its importance declined when the lesion reached the fibrous plaque stage (37 wk on diet). The reason for this may be that the fatty streak formation and growth relies heavily on the recruitment of monocytes macrophages (44), and lipid-filled macrophages are the main component of the fatty streak (Fig. 1 *B*). P-selectin is an excellent candidate to play a role in this recruitment as it was shown to promote recruitment of monocyte/macrophages in thioglycollate-induced inflammation (16), in contact hypersensitivity response (17), and together with E-selectin in skin excisional wounds (44a). It is possible that E-selectin also plays a role in monocytes recruitment to the fatty streak and that the growth of the lesions will be further delayed in mice deficient in both of the endothelial selectins. The normal progression from fatty streak to fibrous plaque occurred in both P-selectin–positive and negative animals, as indicated by the similar intensity of α-actin staining in the lesions of mice of both genotypes (not shown). At 37 wk, we have observed that the lesions in the two genotypes LDLR /P-selectin and LDLR /P-selectin were of comparable size as determined both from sections in the cusp region and by computer analyses of lesion areas in the isolated aortae (Fig. 4 *C* and *D*, and Fig. 6). The lesion size in the cusp region was shown previously to correlate well with the extent of lesions in the rest of the aorta (32). Despite the fact that additional recruitment of monocytes and lymphocytes contributes to lesion growth also at the fibrous plaque stage, the lesion sizes in the P-selectin–deficient animals caught up with those of wild types. This suggests that other adhesion molecules must play a role at these later stage or that they are at least able to replace P-selectin in its absence. The importance of P-selectin may return when the atherosclerotic lesion progresses further to the complex stage where thrombus formation with fibrin and platelet deposition are apparent. P-selectin was shown to be important for leukocyte recruitment into platelet thrombi under flow (Ruggeri et al., unpublished observations) and for fibrin deposition onto vascular grafts (45). The hypothesis that P-selectin may play a role in the complex lesion could not be tested as the known mouse models for atherosclerosis do not appear to progress to this stage (28).

We have not addressed whether it is endothelial P-selectin, platelet P-selectin, or both that promote fatty streak growth. P-selectin on platelets mediates rosetting with monocytes and neutrophils (46, 47). This interaction could bring platelets, with all of their biological activities, into the lesion (2, 48). Furthermore, platelets can contribute cholesterol esters to macrophages (49, 50). Thus it is possible that endothelial P-selectin is responsible for the adhesion of monocytes to the lesion site, whereas platelet P-selectin may contribute to cholesterol accumulation in macrophages in situ. We have previously demonstrated that platelets roll on endothelium in a manner similar to leukocytes (19). This rolling is dependent on endothelial P-selectin providing yet another mechanism for platelet accumulation at sites of vascular lesions.

Chemokine cytokine secretion most likely cooperates with adhesion molecules to magnify the recruitment of monocytes to lesion sites and P-selectin may also play a role in this process. Monocyte binding to endothelial P-selectin in the presence of platelet activating factor induces secretion of monocyte chemotactic protein-1 and tumor necrosis factor-α (TNF-α; 51). Moreover, TNF-α can increase expression of endothelial P-selectin (14), possibly further potentiating monocyte recruitment to the lesion. Perhaps one of the housekeeping functions of endothelial P-selectin is to recruit monocytes/macrophages to scavenge small lipid deposits in the subendothelium. Similar to the situation in chronic inflammation, normal leukocyte recruitment, and vessel wall surveillance may become excessive in a diseased vessel leading to atherosclerotic lesion development.

Atherosclerosis is a polygenic disease. The majority of previously known genes are directly linked to lipid metabolism. We now show that the gene for an adhesion molecule, P-selectin, and likely also those involved in the regulation of its surface expression, could play a role in atherosclerosis.

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EXHIBIT

H

Susceptibility to Infection and Altered Hematopoiesis in Mice Deficient in Both P- and E-Selectins

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Summary

We describe the phenotype of mice lacking both endothelial selectins after sequential ablation of the genes encoding P- and E-selectins. In contrast with the rather mild phenotypes observed in mice deficient in a single selectin gene, the doubly deficient mice present extreme leukocytosis, elevated cytokine levels, and alterations in hematopoiesis. Granulocytopoiesis is increased both in bone marrow and spleen, while erythropoiesis is partially translocated to the spleen. Virtual lack of leukocyte rolling and low extravasation at sites of inflammation make these animals susceptible to opportunistic bacterial infections, to which they succumb. Our results show that the absence of endothelial selectins severely affects leukocyte homeostasis and indicate that these two selectins are as important for normal leukocyte function as are the leukocyte β2 integrins.

Introduction

The emigration of white blood cells to inflammatory sites is believed to require at least four defined steps: leukocyte rolling along activated endothelium, leukocyte activation, firm adhesion, and transendothelial migration (Butcher, 1991; Springer, 1995). Several types of adhesion molecules are involved in this sequential process, but their relative importance remains unclear. The selectin family of genes, clustered on chromosome 1, contains three members with a common structure containing an N-terminal lectin domain (McEver et al., 1995; Tedder, 1995). L-selectin is found constitutively on most leukocytes. Originally recognized as a lymph node homing receptor, it has also been shown to participate in adhesion of leukocytes to endothelium and leukocyte rolling. P-selectin is stored in α granules of platelets and Weibel–Palade bodies of endothelial cells. Rapid translocation to the plasma membrane occurs upon activation. P-selectin is an important mediator of leukocyte rolling. The other endothelial selectin, E-selectin, is not stored, but requires de novo synthesis following stimulation. It is therefore thought to be expressed on the membrane of endothelial cells only under inflammatory conditions. E-selectin also promotes the interaction of leukocytes with endothelium. The role of E-selectin in leukocyte rolling in vivo is controversial (Olofsson et al., 1994; Ley et al., 1995).

In the past three years, mice lacking each of the selectins have been described. P-selectin-deficient mice have virtually no leukocyte rolling upon exteriorization of the mesentery, and the extravasation of neutrophils in these animals is delayed in thioglycollate-induced peritonitis (Mayadas et al., 1993). L-selectin deficiency, in addition to defects in lymphocyte homing to lymphoid tissues, also delays thioglycollate-induced peritonitis and reduces leukocyte rolling (Arbones et al., 1994). Mice lacking E-selectin, in contrast, were reported to have no defect in models such as thioglycollate-induced peritonitis unless anti-P-selectin antibodies were infused, suggesting overlapping functions of P- and E-selectins (Labow et al., 1994). The differences among the three strains of mice lacking each of the selectins show that, despite some overlapping functions, they play distinct roles in vivo.

Two known human clinical syndromes result from deficiencies in leukocyte adhesion molecules. Leukocyte adhesion deficiency type 1 (LAD-1) stems from defective β2 integrins. The disease is manifested by recurrent bacterial and fungal infections without pus formation and deficiencies in wound healing (Anderson and Springer, 1987). LAD-2 results from a defect in the synthesis of fucosylated carbohydrates, among which are the ligands for the selectins (Etzioni et al., 1992). The clinical manifestations of LAD-2 are complex and include developmental defects and recurrent bacterial infections (Frydman et al., 1992). Neutrophilia is conspicuous in both disorders. Further analyses of these rare diseases would be enhanced by development of animal models.

We report here the characterization of P- and E-selectin doubly deficient mice generated by two rounds of homologous recombination in embryonic stem (ES) cells. These mice display a phenotype reminiscent of LAD patients, including defects in leukocyte extravasation at sites of inflammation as well as susceptibility to opportunistic bacterial infections. They also exhibit alterations in hematopoiesis, with elevated levels of hematopoietic cytokines. These phenotypic characteristics reveal broader roles for the endothelial selectins than had been suspected.

Results

Generation of P- and E-Selectin Double-Deficient Mice

The proximity of the genes encoding the selectin family (within 300 kb) precludes production of doubly deficient mice by mating singly deficient animals. Therefore, mutants for both vascular selectins were engineered

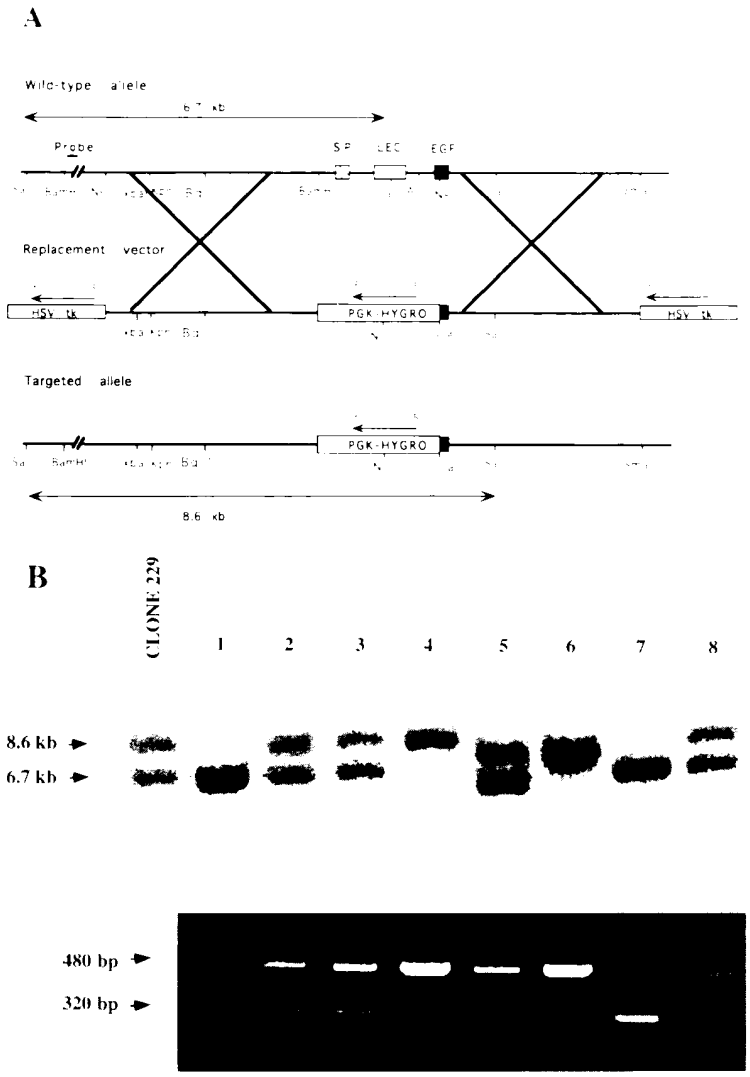

Figure 1. Targeting Strategy and Genotyping of Progeny from Double Heterozygous Crosses

(A) The wild-type E-selectin locus is shown in the upper line. To construct the targeting vector, the hyg cassette was inserted into 7.1 kb of E-selectin sequence. The exons encoding the signal peptide, lectin domain, and part of the EGF domain were deleted in this process. Although HSVtk sequences were included at both ends of the targeting vector, negative selection with gancyclovir was not used owing to toxicity. Resistant clones were screened with a 5′ probe that identifies the targeted 8.6 kb mutant and 6.7 kb wild-type SacI bands. Targeted clones were verified with AccI and NcoI.

(B) Southern blot analysis of E-selectin locus from a SacI digest and PCR analysis for the P-selectin null mutation. Genomic DNA was extracted from tail biopsies of a representative litter. The fragment sizes are indicated. The first lane shows a digest of an ES cell clone, heterozygous for E-selectin, for comparison. The lower panel shows a PCR assay for P-selectin. The 480 bp band represents the targeted allele. These null mutations segregate together during meiosis.

through a second round of gene targeting by homologous recombination in ES cells that were heterozygous for a P-selectin mutation (Mayadas et al., 1993). To make the targeting vector (Figure 1A), we removed a 1.9 kb genomic sequence containing exons encoding the signal peptide, the lectin domain, and part of the epidermal growth factor (EGF) domain and replaced it with a hygromycin B resistance gene driven by phosphoglycerate kinase promoter (PGK-hyg'). The replacement vector included 3.2 kb and 3.9 kb of genomic DNA flanking the PGK-hyg'.

ES cells were electroporated, and 1326 resistant clones were picked. Four homologous recombination events were detected by Southern blot analysis (Figure 1B). Highly chimeric animals were generated from these clones. Two of the four clones transmitted the mutation to their offspring. Germline transmission from one clone produced either P-selectin-deficient heterozygotes or E-selectin-deficient heterozygotes, indicating a recombinational event in *trans*. The progeny transmitted through the germline of chimeras from the second clone were either heterozygous for both P- and E-selectins or of wild-type genotype, indicating the presence of the desired double mutation in *cis*. The resulting doubly heterozygous animals were inter-crossed to obtain viable doubly deficient homozygous mice (Figure 1B).

Double mutants, either heterozygous or homozygous, showed no differences in weight and reproductive ability compared with wild-type littermates. The tail length of these mice was also comparable, suggesting that the development of even the longest blood vessels is not impaired in double-deficient mice. Offspring from intercrosses of heterozygous mice were 26% (110 of 426) double homozygotes, 48% (206 of 426) heterozygotes, and 27% (113 of 426) wild-type, indicating that intact P- and E-selectin genes are not required for embryonic angiogenesis or survival after birth.

Verification of Null Alleles for P- and E-Selectin

We have previously shown that the P-selectin mutation is a null mutation (Mayadas et al., 1993). To test whether intact E-selectin mRNA is produced by P- and E-selectin double mutants (P/E) and E-selectin mutants (E),

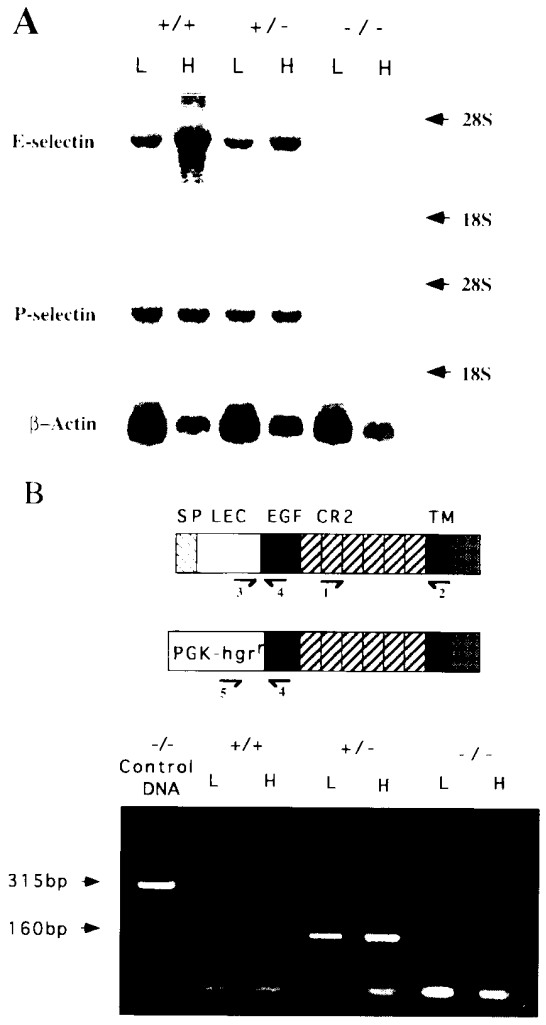

Figure 2. mRNA Analyses of Endothelial Selectins

(A) Northern blot. Total RNA from LPS-treated wild-type (· / ·), double-heterozygous (· /), and double-homozygous (/) mice was isolated from the hearts (H) and lungs (L). Samples were electrophoresed on a 1.2% agarose–0.66 M formaldehyde gel and sequentially hybridized with the rat E-selectin, mouse P-selectin, and mouse β-actin probes. The 28S and 18S bands were comparable after staining the gel with ethidium bromide (data not shown).

(B) RT–PCR analysis. cDNA conversion was achieved from cardiac and pulmonary total RNA of · / · , · / , and / mice using oligo(dT) as primer. A product was obtained using primers downstream to the deletion (primers 1 and 2; data not shown). While a wild-type band (160 bp) was produced from cDNA of · / · and · / mice using a set of three primers (3, 4, and 5), no product was detectable from cDNA of / mice. The mutant band (315 bp), generated by primers 4 and 5, was not detected in · / and / cDNA, showing that no stable readthrough message is produced from the mutated E-selectin locus. The lower band in each lane represents primer dimerization.

we treated mice with lipopolysaccharide (LPS) to induce expression of E-selectin and up-regulation of P-selectin (Bevilacqua et al., 1987; Sanders et al., 1992). Northern blots (Figure 2A) did not show any transcript of wild-type size for either P- or E-selectin in double mutants. A faint 3.0 kb band (Figure 2A) may represent an aberrant message. Rehybridization with a probe from the lectin domain (data not shown) showed no message in P/E animals. No expression of wild-type E-selectin message was seen in E mice (data not shown). We performed reverse transcriptase–polymerase chain reation (RT–PCR) to detect even low levels of E-selectin mRNA. A product from wild-type, E , and P/E mice was obtained using primers from the second complement repeat region and the transmembrane domain (data not shown). However, no cDNA could be amplified from homozygous mutant tissue RNA with primers derived from the lectin and EGF domains (Figure 2B). Hearts from LPS-treated wild-type mice showed positive immunofluorescent staining for both P- and E-selectins in capillaries and small venules, whereas no detectable specific staining for E-selectin or P-selectin was visible in stimulated endothelium of double mutants. Likewise, no E-selectin protein was detectable in E mice with normal P-selectin expression (data not shown).

To verify the expression of L-selectin, leukocytes were labeled with the L-selectin antibody, MEL-14, and anti-α_M antibodies. P/E mice presented increased numbers of neutrophils, all of which expressed normal levels of Mac-1. Two populations of neutrophils were found with regard to L-selectin expression, with about half of the cells showing normal levels and the rest being negative for L-selectin (data not shown). This may be due to shedding of L-selectin by activated or senescent circulating neutrophils. Nonetheless, the L-selectin gene, which lies between the mutated P- and E-selectin genes, remains functional.

P- and E-Selectin Double-Deficient Mice Exhibit Pronounced Leukocytosis and Splenomegaly

To determine whether lack of both endothelial selectins would influence leukocyte counts, peripheral blood cells were quantitated and leukocyte subpopulations were assessed (Table 1). We found a mean 3.9-fold elevation of total leukocytes in the double mutants compared with wild-type counterparts. Mature polymorphonuclear neutrophils were increased more than 16-fold and represented more than 50% of leukocytes. Absolute numbers of monocytes, eosinophils, and lymphocytes were also elevated (Table 1). No immature forms were seen on blood films of P/E animals. The hemoglobin levels and reticulocyte and platelet counts of P/E mice were comparable with those of wild-type littermates. Blood counts were normal (total leukocytes, 5800 · 600; neutrophils, 737 · 150, n 10) in E mice.

To test for leukocytosis throughout the life of double-mutant mice, we harvested blood from neonatal mice (less than 18 hr old) and determined neutrophil numbers. Even at this young age, total leukocyte counts were increased in double-deficient mice, mainly due to a 3.3-fold increase in neutrophil numbers (2720 · 528 [n 9] for wild type; 9010 · 927 [n 13] for P/E ; $p < 0.001$).

These abnormalities suggest a role for both P- and E-selectins in leukocyte homeostasis and imply that E-selectin can be expressed without obvious infection or inflammation. To examine this possibility, we isolated RNA from several organs of healthy wild-type mice. By

Table 1. Peripheral Blood Counts

Blood Cell Types	Wild Type		P/E		Fold Increment	p Value
Total leukocytes (per l)	6000	800	23100	2000	3.9	0.001
Neutrophils	824	91	13625	1450	16.6	0.001
Monocytes	180	25	1757	411	9.8	0.002
Eosinophils	93	24	567	155	6.3	0.009
Lymphocytes	4919	750	7368	619	1.5	0.03
Platelets (10 /. l)	821	103	1021	103	–	0.2
Hemoglobin (g/l)	157	1	158	1	–	0.9
Reticulocytes ( )	2.9	0.4	2.8	0.4	–	0.8

n 8, except for reticulocyte counts, where n 12. P/E / . P- and E-selectin double-deficient mice.

RT-PCR, we found trace levels of E-selectin transcript in every organ studied (Figure 3A); expression appeared highest in the lung and bone marrow. We also examined mRNA by Northern blot of unstimulated heart and lung. Again, a trace of message was detected in both organs (Figure 3B).

We consistently observed splenomegaly in double mutants. Splenic weights, normalized to body weights, from P/E and wild-type mice were increased about 2-fold in favor of the P/E mice. Spleen weights of mice deficient in only P-selectin (P) or E-selectin were comparable with those of wild-type mice. Weights of other lymphoid organs, such as the thymus and peripheral lymph nodes, were also comparable between P/E and wild-type mice. Microscopic examination of spleen sections disclosed an expanded red pulp (Figure 4) and increased extramedullary hematopoietic activity (EMH). Histologic examination of thymus, lymph nodes including Peyer's patches, kidneys, hearts, lungs, brain, intestine, and skeletal muscle showed no obvious abnormalities in the double mutants. Three out of ten P/E animals examined showed evidence of focal EMH in the liver. The abnormalities in the spleens and, in some cases, the livers, which were not seen in mice lacking a single endothelial selectin, indicate a greatly expanded hematopoiesis in mice deficient in both endothelial selectins.

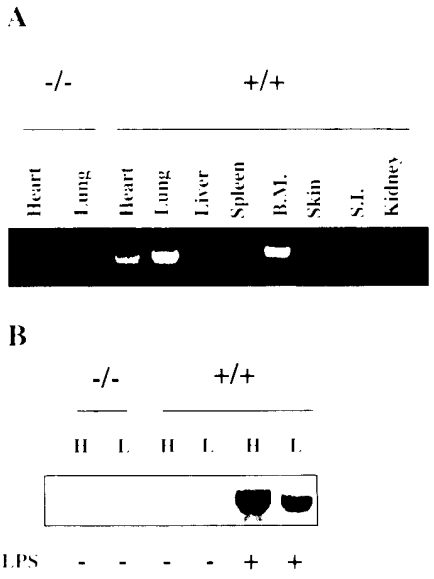

Figure 3. Analyses of E-Selectin mRNA in Tissues of Wild-Type Mice

(A) RT-PCR analysis. RNA was isolated from organs (indicated above) of resting double-deficient (/) and wild-type mice (/). Organs from two / mice were pooled. Reverse transcription was obtained from 4–5 μg of total RNA, and PCR amplification using primers 2 and 3 (see Figure 2B) was performed. A 1.5 kb E-selectin band was detected in every organ studied, while no detectable product was obtained from the lungs and hearts of / mice.

(B) Northern blot. Total RNA of unstimulated hearts (H) and lungs (L) of / and / mice was electrophoresed for Northern blot analysis and probed with the mouse lectin domain sequence. Under resting conditions, trace of E-selectin transcript is detected in the lungs and the hearts of / mice. Note the massive induction of E-selectin in the cardiac and pulmonary tissues of mice treated with LPS.

The Double Mutation Leads to Elevated Cytokines and Alterations of Hematopoiesis

We analyzed hematopoiesis in the doubly mutant mice to seek an explanation for the elevated numbers of white

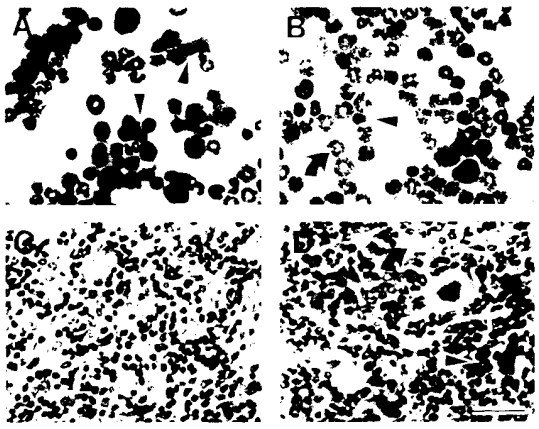

Figure 4. Bone Marrow Smears and Splenic Histology

Femoral bone marrow smears from wild-type (A) and P- and E-selectin double-deficient mice (P/E) (B). Mature neutrophils (arrow) account for the majority of nucleated cells of the double-mutant bone marrow with less numerous erythroid precursors (arrowhead), whereas a normal myeloid to erythroid ratio is seen in the wild-type bone marrow. Spleen sections were stained with hematoxylin–eosin. While the red pulp of wild-type spleens (C) is composed mainly of mononuclear cells, the red pulp of P/E spleens (D) is expanded with large numbers of neutrophils (arrow) and increased hematopoietic activity, as shown by numerous erythroid precursors (arrowhead). Bar, 50 μm.

Table 2. Bone Marrow Differential Counts

Nucleated Cell Types	Wild Type (% ± SEM)	P/E (% ± SEM)	p Value
Myeloblasts	0.6 ± 0.1	0.8 ± 0.2	0.3
Promyelocytes	1.0 ± 0.2	2.4 ± 0.4	0.02
Myelocytes neutrophil	1.5 ± 0.3	3.3 ± 0.2	0.001
Myelocytes eosinophil	0.1 ± 0.1	0.3 ± 0.2	0.4
Metamyelocytes neutrophil	4.1 ± 0.5	8.7 ± 0.9	0.001
Metamyelocytes eosinophil	0.4 ± 0.2	0.9 ± 0.2	0.09
Bands and rings neutrophil	13.9 ± 0.7	20.5 ± 1.9	0.009
Bands and rings eosinophil	0.6 ± 0.2	1.6 ± 0.7	0.2
Polymorphonuclear neutrophils	27.9 ± 1.2	38.6 ± 2.2	0.002
Polymorphonuclear eosinophils	1.7 ± 0.5	2.4 ± 0.6	0.3
Monocytes	0.1 ± 0.1	0.4 ± 0.2	0.3
Lymphocytes	14.9 ± 1.1	9.5 ± 0.9	0.004
Plasma cells	0.0 ± 0.0	0.4 ± 0.2	0.1
Pronormoblasts	0.8 ± 0.2	0.1 ± 0.1	0.003
Basophilic normoblasts	3.6 ± 0.6	1.0 ± 0.3	0.003
Polychromatophilic normoblasts	27.6 ± 2.4	8.9 ± 1.2	0.001
Orthochromic normoblasts	1.0 ± 0.2	0.2 ± 0.1	0.006
Myeloid/Erythroid ratio	1.6 ± 0.2	8.7 ± 1.5	0.001

Differential counts of hematopoietic elements of femoral bone marrows from at least 300 nucleated cells per slide were made. Averages were obtained from six wild-type and six P/E − / − mice.

cells. Femoral bone marrows were extracted from wild-type and healthy-appearing 6-week-old P/E − / − mice to assess their complement of nucleated cells. Neutrophilic granulocytes comprised most of the cells of the P/E − / − bone marrow extracts, with an average of 60% segmented bands and ring forms versus 42% in wild-type mice (Table 2). Although differentiating myeloid precursors were significantly more numerous in the double-mutant mice, blast forms were not increased. In contrast, precursor cells from the erythroid lineage were underrepresented (Table 2). Consequently, the myeloid to erythroid ratio was increased more than 5-fold. The total cellularity of femoral marrows was comparable between wild-type and double-mutant mice ($1.6 \pm 0.1 \times 10^7$ versus $1.9 \pm 0.2 \times 10^7$ nucleated cells, n = 12). Bone marrow morphology and cellular composition were normal in P-selectin-deficient mice (Johnson et al., 1995) and in E − / − mice (data not shown).

To assess myeloid and erythroid progenitor cells, we plated bone marrow and spleen cells into semi-solid media containing appropriate hematopoietic growth factors. Colony-forming units-granulocyte/macrophage (CFU-GM) and erythroid-burst-forming units (BFU-E) were counted after 7 days. In contrast with the bone marrow, the splenic cellularity was increased 2-fold in P/E − / − mice ($1.4 \pm 0.3 \times 10^8$ versus $2.7 \pm 0.2 \times 10^8$; p < 0.005), which parallels the increased spleen weight. Bone marrows from double-mutant and wild-type mice harbored similar proportions of CFU-GM and BFU-E (Table 3), whereas the total numbers of CFU-GM and BFU-E recovered from spleens of P/E − / − mice were significantly increased (Figure 5). We next assayed CFU-GM and BFU-E in 14.5-day-old fetal liver. Numbers of CFU-GM and BFU-E were not significantly different between wild-type and P/E − / − mice, suggesting that the abnormalities in hematopoiesis were acquired after birth (Table 3).

The observed increased hematopoietic activity in adult P/E − / − mice might be a response to systemic elevation of hematopoietic cytokine production in this genotype. We therefore assayed two hematopoietic cytokines, granulocyte/macrophage colony-stimulating factor (GM-CSF) and interleukin-3 (IL-3), in sera of wild-type, P − / −, and P/E − / − mice. While there was a trend toward increased levels of cytokines in mice deficient in P-selectin only, doubly deficient animals presented, on average, a greater than 40-fold elevation in levels of IL-3 relative to wild-type mice, and GM-CSF levels were increased 5-fold (Table 4).

Occurrence of an Ulcerative Cutaneous Infection in P- and E-Selectin Double Mutants

The consistent findings of leukocytosis and splenic enlargement suggested the possibility of an infection in these P/E − / − mice that are potentially immunocompromised. We first sought pathogenic microorganisms in three P/E − / − mice aged 8–12 weeks. Blood cultures (for bacteria and fungi), extensive viral serologies, and a search for parasites all returned negative. These results suggested that the above abnormalities were not secondary to a detectable infectious process. We subsequently noticed, however, an increased incidence of a perinasal cellulitis with or without ulceration in double-deficient mice. In a cohort of 388 wild-type (n = 97), double-heterozygous (n = 190), and double-homozygous (n = 101) mice, 11 double mutants developed this condition (all aged >8 weeks), whereas none of the heterozygotes or wild-type littermates did at a median age of approximately 11 weeks. The prevalence of the skin disorder increases with age, the majority of the mice older than 4 months being affected. Afflicted mice presented very high leukocyte counts (73,200 ± 8,900, n = 6), which is severalfold higher than that seen in asymptomatic P/E − / − mice (Table 1). This chronic cellulitis, which becomes ulcerative, may evolve to the anterior cervical and upper thoracic regions (Figure 6A) or to periorbital areas, producing conjunctivitis. Fatalities

Table 3. Hematopoietic CFU

	Wild Type		P- and E-Selectin /	
Colonies	CFU-GM	BFU-E	CFU-GM	BFU-E
Fetal liver (number of colonies/10 nc)	140 · 9	38 · 2	134 3	32 · 3
Bone marrow (number of colonies/10 nc)	3940 · 388	247 · 49	4831 377	307 · 46
Spleen (number of colonies/10 nc)	137 · 25	74 · 17	331 · 61	108 · 24

Femoral bone marrow and splenic cells were isolated from 6- to 8-week-old mice, and fetal liver cells were obtained from day 14.5 fetuses. After disruption to single cells, the cellularity was determined by counting nucleated cells (nc).
p 0.02, compared with wild-type mice; n 6 in each group.

were observed in mice older than 3 months. Opportunistic bacteria (most commonly Staphyloccocus xylosus and Streptococcus viridans) have been recovered from skin cultures of these animals. When compared with histologic sections of another chronic ulcerative dermatitis occurring in C57BL/6 mice (obtained from Dr. R. Bronson, School of Veterinary Medicine, Tufts University), neutrophil numbers at the lesion sites of P/E mice were markedly reduced. The low number of neutrophils in lesions was also reflected by the histology of draining lymph nodes. These showed only few small germinal centers interspersed with sheets of plasma cells and were devoid of macrophages and neutrophils, which normally occupy the interfollicular space of lymph nodes draining suppurative lesions. Such lymph nodes contrast with those of healthy-appearing P/E mice and may result from the impaired extravasation of neutrophils.

To establish a causal relationship between lesions and bacteria, a cohort of healthy-appearing double-mutant mice aged 5–7 weeks were given broad spectrum antibiotics for 8 weeks. Control P/E littermates received regular water for the same period of time. While five of 12 control P/E mice developed the skin infection (including one death), none of 11 prophylactically treated littermates did. The high prevalence and the severity of this infection in mice deficient in both endothelial selectins, not observed in our colonies of mice lacking either P-selectin or E-selectin, suggest that double-deficient mice present some degree of immunodeficiency.

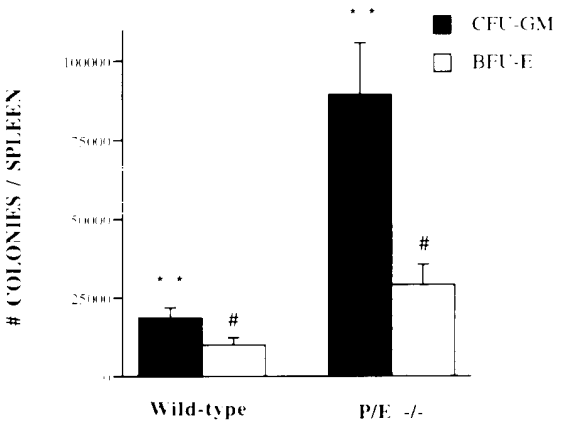

Figure 5. Culture Assays of CFU in the Spleen

Nucleated cells from spleens were isolated, and absolute numbers of CFU-GM and BFU-E were determined. CFU-GM and BFU-E were, respectively, 4.8-fold and 2.9-fold increased in P/E relative mice to wild-type mice. Double asterisks, p 0.003; number sign (#), p 0.03.

Leukocyte Rolling in Inflamed Venules Is Severely Impaired in P- and E-Selectin Double-Deficient Mice

To evaluate the role of P- and E-selectins in leukocyte rolling under inflammatory conditions, we treated mice with tumor necrosis factor α (TNFα) 3.5 hr prior to intravital microscopy of mesenteric venules (Table 5). Although baseline rolling is virtually absent in P-selectin-deficient mice (Mayadas et al., 1993), many rolling leukocytes can be observed after stimulation with TNFα (Ley et al., 1995). However, the rolling flux was significantly lower in our analysis of P mice as compared with wild-type animals (p 0.001). The rolling in E mice after TNFα treatment was not reduced relative to wild type (data not shown). Double mutants showed a 46-fold reduction in rolling compared with wild-type mice ($p < 0.001$) and a 20-fold decrease relative to animals lacking P-selectin ($p < 0.001$), demonstrating that both P- and E-selectins contribute significantly to leukocyte rolling in inflamed venules. In P mice stimulated with TNFα, the affinity of leukocytes for inflamed venules seemed enhanced compared with wild-type controls, as shown by their lower velocity (p 0.02) and shape distortion, forming a uropod-like structure (Figure 7). In contrast, the velocity of the rare rolling leukocytes seen in P/E mice was, on average, much greater than in animals of the other two genotypes (p 0.001). The number of cells adherent to the venular wall, while similar in wild-type and P-selectin knockout mice, was lower in the double mutants ($p < 0.001$), indicating a major combined role for P- and E-selectins in arrest of leukocytes after TNFα stimulation.

Table 4. Hematopoietic Cytokine Levels

Cytokine	Wild Type	P	P/E
IL-3 (pg/ml)	7.4 · 2.1	12.1 · 3.0	317 · 140
GM-CSF (pg/ml)	0.4 · 0.2	1.2 · 1.1	2.1 · 0.8

n 12 for wild type and P/E ; n 8 for P/E .
p 0.05 versus wild type.

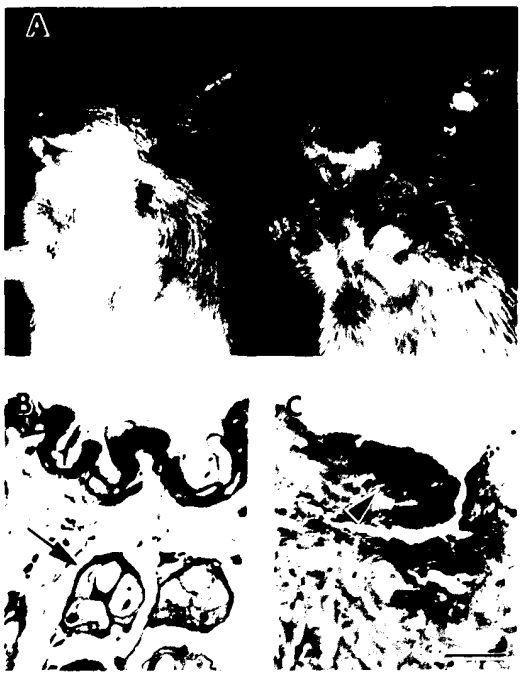

Figure 6. Ulcerative Dermatitis in P- and E-Selectin Double-Deficient Mice

(A) Photograph showing the ulcerative dermatitis in the P/E mice (right) not found in wild-type animals (left). Gram stain of histologic section of wild-type (B) perinasal cutaneous tissue showing a normal epidermis and dermis compared with the ulcerated skin of P/E mice (C), whose epidermis is replaced by a serocellular crust containing large clusters of gram-positive cocci (arrowhead). The normal structures of the skin such as hair follicles (arrow) are no longer recognizable. Bacterial cultures of the tissues of both mice (B and C) revealed S. xylosus organisms. Bar, 50 μm.

Neutrophil Influx in Chemically Induced Peritonitis Is Compromised in Mice Lacking Both Endothelial Selectins

P-selectin-deficient mice exhibit delayed extravasation of neutrophils to inflamed peritoneum compared with wild-type mice (Mayadas et al., 1993). This difference, maximal in the first 2 hr after inflammatory stimuli, narrows at the 4 hr timepoint, possibly owing to E-selectin expression. To investigate whether E-selectin plays a role in the recruitment of neutrophils to inflamed sites and to determine the cooperative function of the two endothelial selectins in this activity, we intraperitoneally injected mice with thioglycollate. The rate of extravasation of neutrophils in P/E mice paralleled that of P mice in the first 4 hr (Figure 8). However, at the 8 hr timepoint, double mutants exhibited a greater than 5-fold reduction compared with wild-type mice ($p < 0.001$) and a 2.5-fold reduction relative to P-selectin knockouts (p 0.02). The defect in neutrophil emigration occurred despite several times more circulating neutrophils in the double mutants. These results are consistent with the inhibition of neutrophil recruitment by anti-P-selectin antibody in E-selectin null mice (Labow et al., 1994).

Discussion

Deficiency in Both Endothelial Selectins Markedly Perturbs Leukocyte Homeostasis

The elevation in most classes of peripheral blood leukocytes in the P/E mice (Table 1) is significantly greater than that reported in other strains of mice deficient in various vascular adhesion molecules (Mayadas et al., 1993; Sligh et al., 1993; Arbones et al., 1994; Xu et al., 1994). Neutrophil numbers are elevated only 1- to 4-fold in these singly deficient animals. In particular, mice deficient in individual selectins show mild neutrophilia in the case of P mice (2.4-fold; Mayadas et al., 1993) or no increase in the case of L (Arbones et al., 1994) or E mice (our data). The 16-fold elevation in blood neutrophils in the P/E mice, first, indicates cooperative functions of the two endothelial selectins and, second, suggests that these two selectins play central roles in leukocyte homeostasis.

The increased leukocyte numbers likely arise from several mechanisms (Figure 9). Some of the elevation presumably follows from elevated cytokine levels and subclinical infections (see below). However, the fact that overtly healthy animals show elevated neutrophil counts, and even newborn pups exhibit a 3.3-fold increase, suggests that some increase in neutrophils is a direct consequence of the absence of P- and E-selectins and is independent of bacterial infections. We have shown greatly reduced leukocyte rolling (Table 5) and extravasation (Figure 8) in the P/E mice and have reported previously that in P mice, in which rolling and extravasation are less severely compromised (Table 5; Figure 8; Mayadas et al., 1993), there is an increase in neutrophil half-life ($t_{1/2}$) in the circulation (Johnson et al., 1995). The P/E mice might, therefore, be expected to show at least an equivalent increase in $t_{1/2}$, with an accompanying increase in numbers of circulating leukocytes.

A function for P- and E-selectins in regulating the

Table 5. Leukocyte Rolling on TNFα-Stimulated Venules

Rolling Characteristics	Wild Type	P	P/E
Number rolling (per minute)	18.4 · 1.8	8.0 0 9	0 4 · 0.2
Number adherent (per 100 μm)	6.7 · 0.8	5.7 0 7	1 1 · 0.4
Leukocyte velocities (μm/s)	8.2 · 1.7	2.7 0 4	25 9 · 3.3
Shear rates (s)	492 · 43	516 56	561 · 102
Venular size (μm)	31.1 · 1.9	33.7 2 2	32 1 · 2.0

Mice were prepared 3.5 hr after administration of TNFα. The number of rolling leukocytes was quantitated by counting the cells passing through a perpendicular plane in 1 min. Averages were obtained from ten counts for wild-type or P-selectin-deficient mice (P) and from the entire filming period for P- and E-selectin double-deficient mice (P/E).

n 3, owing to rare leukocyte rolling; elsewhere, n 6–7.

Figure 7. Leukocyte Rolling in TNF -Stimulated Mesenteric Venules

Mice were treated with TNF 3.5 hr prior to intravital microscopy of the mesentery. Most leukocytes rolling in wild-type venules maintained their round shape. Adherent leukocytes are indicated by arrowheads. The rolling velocity in P-selectin-deficient venules (P) was reduced (see Table 5), with frequent formation of uropod-like structures (arrow) suggesting cellular activation. Leukocyte rolling was nearly absent, and the number of adherent cells was markedly reduced, in P- and E-selectin double-deficient mice (P/E). The blood flow is from top to bottom. Bar, 20 μm.

levels of leukocytes independent of infection or inflammation is unexpected. Both of these selectins have previously been reported to be elevated only on activated endothelium. We report here that low levels of E-selectin mRNA can be detected in many tissues of healthy wild-type mice (Figure 3), adding further support to the argument that E-selectin, together with P-selectin, plays an unsuspected role in normal homeostasis of leukocytes. Basal expression of endothelial selectins, either constitutive or in response to normal bacterial flora (or both), apparently is essential for maintaining normal leukocyte levels.

Rolling and adherence of leukocytes in response to such basal expression, or at higher levels after infection or inflammation, is clearly dependent on the endothelial selectins, at least in mesenteric venules (Table 5). The reduced extravasation of neutrophils into the skin and the development of infectious ulcerative dermatitis in P/E mice (Figure 6) indicate that the same is true even in the small vessels of the skin, where shear rates are lower than in other vascular beds. It has been suggested

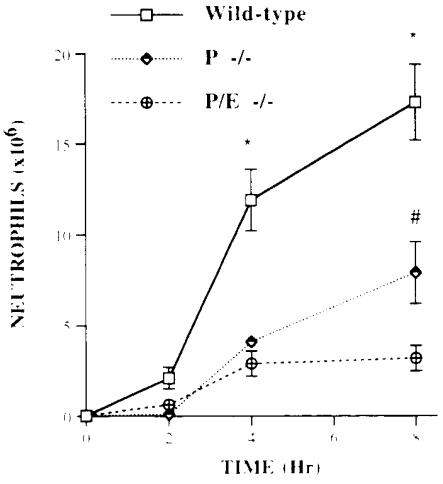

Figure 8. Peritoneal Neutrophil Influx after Thioglycollate Injection

Peritoneal lavages were performed at intervals following thioglycollate administration; total cell numbers were determined with a hemocytometer; and the percentage of neutrophils was obtained. Four to ten animals of each genotype were used per timepoint except for 0 hr (n 3). Asterisk, $p < 0.001$; number sign (#), p 0.02, compared with P/E .

that selectin-mediated rolling might not be necessary under conditions of low shear stress (Yamada et al., 1995). The phenotype of these mice shows that selectins are indeed necessary for effective rolling, adhesion, and extravasation and that their functions cannot be replaced by integrins. Furthermore, our results show that L-selectin expressed on leukocytes cannot substitute for the endothelial selectins. Although L-selectin has been implicated in rolling (Ley et al., 1991; Arbones et al., 1994), an inducible ligand for L-selectin (unless it is one of the endothelial selectins, as suggested by Picker et al. [1991]) is not sufficient to mediate rolling after TNFα activation (Table 5) or for adequate extravasation in response to peritoneal inflammation (Figure 8) or bacterial infection (Figure 6). L-selectin-deficient mice show no elevations in circulating leukocytes and are not subject to spontaneous bacterial infections, although they do show deficiencies in leukocyte recruitment to induced inflammatory sites (Arbones et al., 1994). Taken together, the results on P/E and L mice suggest a primary role for endothelial selectins in response to infection and inflammation. In contrast, homing of lymphocytes to peripheral lymph nodes is markedly depressed in L mice (Arbones et al., 1994), resulting in small nodes with few germinal centers, while the size and morphology of peripheral lymph nodes appear normal in our P/E mice; L-selectin appears primary in this aspect of leukocyte traffic. The defect in leukocyte homeostasis in the P/E mice is as strong as that in LAD-1 patients lacking β2 integrins, indicating that rolling mediated by endothelial selectins is as important as adhesion mediated by β2 integrins. Perhaps leukocyte rolling on endothelial selectins contributes to the leukocyte activation necessary for adhesion. This idea gains some support from recent results showing a necessary role for P-selectin together with platelet-activating factor in monocyte activation (Weyrich et al., 1995) and from the activated appearance of leukocytes rolling on E-selectin alone (Figure 7).

Animals Deficient in Both P- and E-Selectins Are Susceptible to Opportunistic Skin Infections

The central importance of P- and E-selectins acting together is further underlined by the high prevalence of spontaneous bacterial dermatitis in mice lacking both (Figure 6). The prevention of this dermatitis by oral antibiotics indicates that these life-threatening infections are

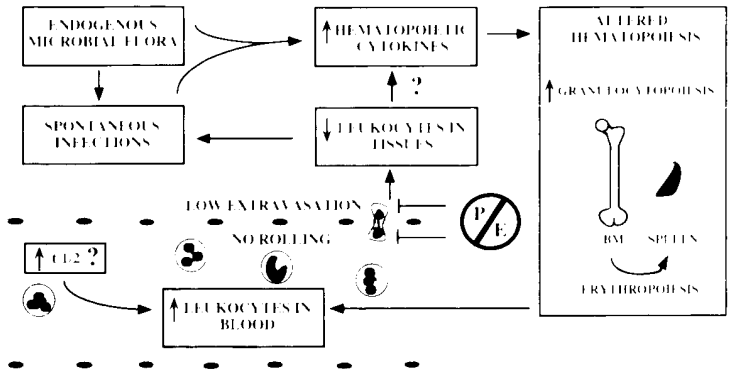

Figure 9. Schema Illustrating Putative Pathways Causing Alterations in Leukocyte Homeostasis in Mice Lacking Endothelial Selectins

Absence of both endothelial selectins drastically reduces rolling and extravasation of leukocytes. This produces elevation of blood leukocyte numbers, probably as a consequence of increased half-life in the circulation and reduced numbers of tissue leukocytes. Cytokine levels rise, probably from a variety of causes: increased growth of endogenous bacterial flora, higher blood leukocyte levels releasing cytokines, and, possibly, feedback stimulation owing to the reduced numbers of tissue leukocytes. The elevated cytokines cause increases and alterations in hematopoiesis, further elevating the blood leukocyte numbers. Despite the excessive numbers of leukocytes in blood, tissue leukocyte numbers remain low, and subclinical bacterial infections progress to become life threatening.

not secondary to nonbacterially derived (i.e., autoimmune) inflammation and are due to opportunistic infections by normal bacterial flora. In other words, the absence of endothelial selectins produces a state of immunodeficiency, reminiscent of LAD-1 and LAD-2. Such spontaneous infections have not been observed in other strains of mice deficient in vascular adhesion molecules, including mice lacking individual selectins (Mayadas et al., 1993; Arbones et al., 1994; Labow et al., 1994) or mice largely lacking β2 integrins (Wilson et al., 1993) or their ligand, intercellular adhesion molecule 1 (Sligh et al., 1993; Xu et al., 1994).

The development of spontaneous infections in the skin is likely a direct consequence of the markedly reduced leukocyte rolling and extravasation in the P/E mice (Figures 6 and 8). This presumably leads to reduced numbers of tissue leukocytes, which would normally block progression of these infections (Figure 9). Clearly, either P- or E-selectin is sufficient to preclude clinical infection, while L-selectin acting alone is not, and neither are β2 integrins, with or without cooperating β1 integrins. These results suggest that absence of ligands for endothelial selectins is likely the major contributor to the infectious aspects of the LAD-2 syndrome. They also raise concerns about the possible side effects of long term use of anti-inflammatory drugs that target both endothelial selectins. The further elevation of circulating leukocytes in the infected mice resembles that seen in both LAD syndromes during infectious episodes and presumably reflects elevated granulopoiesis in response to elevated cytokine levels induced by the developing bacterial infection (Figure 9).

Absence of Endothelial Selectins Causes Alterations in Hematopoiesis

Both myeloid precursors and hematopoietic cytokines were significantly elevated in overtly healthy P/E mice (Tables 2–4). The elevation of cytokine levels could arise from several causes (Figure 9). Elevated numbers of circulating leukocytes could themselves release higher levels of cytokines than normal. Alternatively, reduction in tissue leukocytes could trigger a positive feedback loop, inducing enhanced granulopoiesis. Such positive feedback loops have been invoked for other hematopoietic lineages (Schooley and Mahlmann, 1972; Odell et al., 1979; Bartocci et al., 1987).

Since CFU-GM and BFU-E are not elevated in fetal liver, it appears that most or all of the alterations in hematopoiesis arise postnatally, perhaps in response to subclinical bacterial infections (Figure 9). In support of this possibility, circulating leukocytes are even further increased in animals showing obvious signs of infection. The bone marrow exhibits a major increase in cells of the myeloid lineage, although not in the earliest precursors, CFU-GM and myeloblasts (Tables 2 and 3; Figures 4 and 5), whereas the erythroid lineage is underrepresented (Table 2). It has been reported previously that erythropoiesis is elevated in the spleen and elsewhere when myelopoiesis in the bone marrow is enhanced (Molineux et al., 1990). Such is the case in the P/E mice, which show elevated BFU-E in the spleen and EMH in the liver. CFU-GM were also elevated in the spleen. Thus, a plausible explanation for the altered patterns of hematopoiesis is that elevation of cytokines leads to enhanced granulocytopoiesis in the bone marrow and spleen and to displacement of erythropoiesis to the spleen and liver (Figure 9).

However, it is also possible that the endothelial selectins participate directly in homing, proliferation, or differentiation of hematopoietic cells. Primitive hematopoietic progenitors have been reported to bind P-selectin and to express P-selectin glycoprotein ligand-1 (PSGL-1) (Zannettino et al., 1995). Since PSGL-1 is a signaling molecule regulating cytokine production in monocytes (Weyrich et al., 1995) and is a common ligand for both P- and E-selectins (Asa et al., 1995), it might have a role in regulation of hematopoietic cell functions. Notably, Banu et al. (1995, Blood *86*, abstract) recently reported increased numbers of IL-3-responsive megakaryocyte progenitors in the bone marrow of P mice compared with wild-type controls, suggesting the possibility of negative regulation of hematopoietic progenitors by interactions between selectins and their ligands. It is also worth noting that we detect significant levels of E-selectin mRNA in bone marrow of normal wild-type mice (Figure 3). The alterations in hematopoiesis in mice lacking endothelial selectins may therefore originate from several mechanisms leading to either a primary or a secondary dysregulation of hematopoiesis.

Altered hematopoiesis has not been reported in mice deficient in other vascular adhesion receptors. Indeed, the rather modest elevations in circulating cells

have not prompted such investigations. However, more subtle defects in distributions and development of hematopoietic precursors might well be revealed by further analyses of those mice, and given our results radical alterations in hematopoiesis are to be expected in LAD patients.

In conclusion, the phenotypic alterations in mice deficient in both endothelial selectins reveal unanticipated functions of these receptors. They play complementary roles in the regulation of several aspects of leukocyte dynamics even in the absence of obvious infection, inflammation, or vascular injury and even more so when these occur. Absence of these two selectins is not compensated by the continued presence of L-selectin or other adhesion receptors and leads to the early death of the deficient mice. It appears that their functions are as crucial as those of β2 integrins and, indeed, are essential for effective action of the integrin class of receptors in leukocyte adhesion.

Experimental Procedures

Construction of E-Selectin Targeting Vector

Amplified λ phage libraries made from the 129/Sv mouse strain (gift from Drs. H. Wu and R. Jaenisch, Massachusetts Institute of Technology; Stratagene) were screened with a 289 bp PCR product from the lectin domain of mouse E-selectin. The genomic clones were subcloned into pBluescript (Stratagene). The XhoI–ClaI PGK-hyg' cassette (XhoI blunted) was inserted between the 3.2 kb NsiI–BamHI (BamHI blunted) and 3.9 kb NsiI–SmaI genomic sequences. The resulting 9.1 kb fragment was inserted between two HSVtk cassettes (see Figure 1A). The final 17.5 kb construct was linearized with NotI for transfection.

Cell Culture, Transfection, and Selection

D3 ES cells, clone 32, heterozygous for a P-selectin null mutation were cultured on mitotically inactivated mouse embryonic fibroblasts (MIMEF) in standard ES cell medium (Mayadas et al., 1993). ES cells were electroporated (240 V, 500 μF) with 25 μg of linearized DNA construct and plated on STO feeder cells (Sawai et al., 1991) resistant to hygromycin B in ES cell medium with increased LIF ($2 \cdot 10^3$ U/ml). Hygromycin B (150 μg/ml) was added 24 hr post-transfection. Clones were picked from days 6 to 9 and grown without selection drugs on MIMEF.

Southern Blot Analysis to Identify Targeted Clones

Genomic DNA was isolated from ES cells (Mayadas et al., 1993) and digested with SacI, and fragments were separated on a 1% agarose gel. The DNA was transferred to nylon membrane (Zeta-Probe). A 600 bp MscI–BamHI genomic fragment upstream to the targeting construct was used as a probe. Blots were prehybridized and hybridized in 0.75 M sodium phosphate (pH 7.0), 1 mM EDTA, 7% SDS, 1% bovine serum albumin (BSA), 100 μg/ml salmon sperm DNA at 65 C for 12–18 hr. Filters were then washed twice in 50 mM sodium phosphate, 1% SDS, 1 mM EDTA, 0.5% BSA and twice in the same solution without BSA at 65 C. DNA from positive clones was probed with hyg' to ensure a single integration of the targeting vector. The integrity of the previous P-selectin mutation was assessed by analyzing BamHI genomic digests (Mayadas et al., 1993).

Generation of Chimeric Mice and Genotyping of Progeny

Chimeric animals were prepared as described previously (Mayadas et al., 1993), and genomic DNA of F1 generation was analyzed by hybridizing sequentially Southern blots of BamHI digests with the E-selectin, P-selectin, hyg', and neomycin probes as described above. Genotyping of subsequent generations was performed by PCR assays for P-selectin, E-selectin, or both. The P-selectin assay used forward primers from exon 3 of murine P-selectin (5′-TTG TAA ATC AGA AGG AAG TGG-3′) and from the PGK promoter of the neo cassette (5′-CAC GAG ACT AGT GAG ACG TG-3′) with a reverse primer (5′-AGA GTT ACT CTT GAT GTA GAT CTC C-3′), yielding 322 bp wild-type or 479 bp mutant fragments (or both). The PCR assay for E-selectin used the same PGK forward primer with a forward primer from the lectin domain of mouse E-selectin (5′-GGA CTG TGT AGA GAT TTA CAT CC-3′) and a reverse primer from the EGF domain (5′-GCA GGT GTA ACT ATT GAT GGT-3′) producing a 664 bp wild-type or 315 bp mutant fragments (or both). PCR conditions were 40 cycles of 94 C for 1 min, 60 C for 2 min, and 72 C for 3 min.

Northern Blot and RT-PCR Analysis

Mice were treated with LPS from Escherichia coli O55:B5 (Difco) at 20 μg per gram of body weight 4 hr prior to harvesting the lungs and hearts for total RNA extraction (RNA Stat-60, Tel-test "B"). We electrophoresed 30 μg of total RNA on a 1.2% agarose gel containing 0.66 M formaldehyde. After RNA transfer to nylon membrane, E-selectin transcript was detected with an EcoRI–SphI 1.3 kb rat E-selectin cDNA fragment (Fries et al., 1993) provided by Dr. T. Collins (Brigham and Women's Hospital, Boston, MA). Prehybridization and hybridization conditions were as described above, except that the washing temperature was 45 C. Detection of P-selectin mRNA was performed with a 1.7 kb fragment of the 3′ end of mouse P-selectin cDNA (base pairs 1406–3075), a gift from Dr. D. Vestweber (Max-Planck Institut für Immunobiologie, Freiburg, FRG; Weller et al., 1992). For RT-PCR assays, first strand cDNA conversion of 3–5 μg of total RNA used MoMLV reverse transcriptase (GIBCO) and oligo(dT) as primer. One tenth of the reaction volume served as template for standard PCR with oligonucleotides from the second complement repeat (5′-AAA TCC TGG GAG CTA CCC-3′) and the transmembrane domain (5′-CAG GAG TGA GGT TCC TGC-3′) of E-selectin.

Immunofluorescence Staining, Flow Cytometry, and ELISA

Cardiac tissues were snap frozen. Cryostat sections (5 μm) were fixed in 3.7% (v/v) formaldehyde and permeabilized in 0.5% Triton X-100 in phosphate-buffered saline (PBS). Tissue sections were incubated for 30 min with rat polyclonal antibody to mouse E-selectin (provided by Dr. B. Wolitsky of Hoffmann–La Roche) 1:100, followed by a 30 min incubation at 37 C with fluorescein-conjugated goat antibody to rat IgG (Cappel) 1:100. Leukocytes in whole blood were stained for flow cytometry for 30 min at room temperature with fluorescein-conjugated rat monoclonal antibody to L-selectin (MEL-14; Pharmingen) 1:50 and phycoerythrin-conjugated rat monoclonal antibody to the mouse α_M subunit (Boehringer–Mannheim) 1:50 or phycoerythrin-conjugated rat monoclonal antibody to mouse myeloid differentiation antigen (Gr-1) 1:50. Analysis of 150,000 events was performed on FACSCAN flow cytometer (Becton–Dickinson). Enzyme-linked immunosorbent assays (ELISAs) for IL-3 and GM-CSF were performed as suggested by the manufacturer (Endogen).

Blood Counts, Bone Marrow Analysis, Histology, and Antibiotic Administration

Blood was obtained by retro-orbital venous plexus sampling in polypropylene tubes containing EDTA. Blood from neonatal mice was harvested by severing the jugular vein with fine scissors and was collected as above using heparin-coated capillary tubes. Complete blood counts were determined using an automatic cell counter (Coulter) and differential counts on Wright-stained smears (Harleco). Reticulocyte counts were obtained by staining blood for 10 min using a 1:1 dilution of 5 g/l methylene blue solution. The percent of reticulocytes was assessed by counting 1000 erythrocytes from smears. Femoral bone marrows were flushed with 50 μl of PBS with 10 mM EDTA, and smears from the cell suspension were made. Formalin-fixed tissues were paraffin embedded, sectioned, and stained with hematoxylin–eosin. To prevent the occurrence of bacterial dermatitis, mice were treated orally with trimethoprim (24 mg/dl) and sulfamethoxazole (120 mg/dl) in water for 8 weeks.

Hematopoietic Colony Formation Assays

Femurs were dissected and washed in sterile cold Hanks' balanced salt solution (HBSS). Both ends were trimmed, and the marrow plug

was aseptically flushed with 1 ml of minimum essential medium (MEM$_\alpha$) containing 2% FBS, 100 U/ml penicillin, and 100 μg/ml streptomycin (MEM complete medium), using a 21 gauge needle. A single cell suspension was obtained by gently aspirating several times with the same needle and syringe. Spleens were removed and washed in HBSS, and cells were extracted through a stainless steel grid. Cells from both organs were added at a 1:10 (v/v) ratio in Methocult GF M3434 (Stemcell Technologies). Bone marrow and spleen cells were plated at a density of 1.5 · 10^5 or 1 · 10^5 cells per dish, respectively, in duplicate assays. For fetal hematopoietic progenitors, 14.5-day-old livers from wild-type and P/E mice were disrupted to single cells by passing through 18, 21, and 26 gauge needles. Cells were washed in MEM complete medium, and 5 · 10^4 cells were plated per dish.

Analysis of Leukocyte Rolling by Intravital Microscopy

Mice were treated with murine TNF$_\alpha$ (Genzyme), 0.5 μg in 500 μl of PBS, intraperitoneally. The mesentery was prepared (Johnson et al., 1995), and venules 28–40 μm in size were recorded for 20 min. Centerline erythrocyte velocity (V_{rbc}) was measured using an optical Doppler velocimeter (Microcirculation Research Institute, Texas A & M College of Medicine, TX). Venular shear rate (τ) was calculated based on Poiseuille's Law for a Newtonian fluid: $\tau = 8(V_{mean}/D_v)$, where D_v is the diameter of the venule and V_{mean} is estimated from the measured V_{rbc} using the empirical correlation $V_{mean} = V_{rbc}/1.6$ (Baker and Wayland, 1974). The number of rolling leukocytes per minute was determined by taking five counts of 1 min in the first 10 min of filming and a second set of five counts during the latter half of recording. The velocity of leukocyte rolling was determined by measuring, field by field, the distance traveled in a given time by 20 consecutive leukocytes. Cells were considered adherent if they remained stationary for >30 s and were counted over a venular segment of 100 μm.

Thioglycollate-Induced Peritonitis

Mice were injected intraperitoneally with 1 ml of 2.95% thioglycollate (Sigma), and peritoneal lavages and leukocyte determinations were performed as previously described (Mayadas et al., 1993).

Statistical Analysis

Data are presented as mean ± SEM. Statistical significance was assessed by Student's t test.

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EXHIBIT

J

Blood Cell Dynamics in P-Selectin–Deficient Mice

By Robert C. Johnson, Tanya N. Mayadas, Paul S. Frenette, Reina E. Mebius, Meera Subramaniam, Ann Lacasce, Richard O. Hynes, and Denisa D. Wagner

P-selectin is expressed on the surfaces of activated platelets and endothelium where it mediates binding to leukocytes. P-selectin–deficient mice were shown to exhibit peripheral neutrophilia (*Mayadas et al: Cell 74:541, 1993*). We now show that this is not caused by changes in bone marrow precursors nor by a lack of neutrophil margination. Both P-selectin–positive and –negative animals displayed similar increases in peripheral blood neutrophil numbers after injection of epinephrine. However, clearance of ^{51}Chromium-labeled neutrophils is delayed in mice deficient for P-selectin, indicating that the neutrophilia is at least in part the result of delayed removal. We detected no obvious alterations in lymphocyte differentiation, distribution, or adhesion to high endothelial venules in peripheral lymph nodes. Through intravital microscopy, we examined the impact of P-selectin deficiency on leukocyte/endothelial interaction beyond the initial stages of inflammation. Four hours after the administration of an inflammatory irritant, leukocyte rolling was observed even in the absence of P-selectin. There were significantly fewer rolling cells relative to wild-type mice, and their velocity was reduced. Moreover, in the peritonitis model, the number of peritoneal macrophages in wild-type mice increased threefold at 48 hours, whereas the macrophages in the mutant mice remained near baseline levels. Thus, whereas P-selectin is known to be involved in early stages of an inflammatory response, our results indicate that it is additionally responsible for leukocyte rolling and macrophage recruitment in more prolonged tissue injury.

THE SELECTIN FAMILY of vascular adhesion receptors mediates the early steps in leukocyte adhesion to endothelium and platelets.[1-3] Adhesion is mediated by the selectins' lectin domain and a specific carbohydrate moiety that is part of the ligand molecule L-selectin, present on leukocytes, mediates lymphocyte homing to peripheral lymph nodes, and together with the endothelial selectins P- and E-selectin, is involved in leukocyte extravasation at sites of inflammation. The roles of the individual selectins in leukocyte extravasation are unclear. In some situations their functions may be overlapping.[4] P-selectin, which was first identified in platelets,[5,6] is constitutively present in storage granules of platelets and also of endothelial cells.[7-10] It is thought to be involved in acute responses as the contents of the alpha-granules of platelets and the Weibel-Palade bodies of endothelial cells can be rapidly released upon cellular activation. E-selectin in contrast is thought to be involved only in later stages of inflammation as its expression is induced by de novo syntheses stimulated by inflammatory cytokines.[11] Recently, the synthesis of P-selectin was also shown to be upregulated by cytokines[12,13] and in addition, after surface expression on the endothelial cells, P-selectin can return to nascent Weibel-Palade bodies and it may be reexported to the cell surface.[14] Therefore, a role for P-selectin in more chronic processes cannot be excluded.

To evaluate the role of P-selectin in homeostasis and disease, we prepared an animal model lacking P-selectin by gene-targeting technology.[15] In this model we have established the expected role of P-selectin in acute injury and early inflammation. The P-selectin–deficient mice have twofold to threefold elevated peripheral neutrophil counts in comparison to wild-type mice.[15] This observation remains unexplained because detailed analysis of bone marrow (BM) precursor cells was not performed nor was the clearance of circulating neutrophils examined. Therefore, we decided to further study the blood cell dynamics in the P-selectin–deficient mice and we report here that neutrophil clearance is reduced in the mutant mice.

Although exteriorization of the mesentery induces rolling of leukocytes on the endothelium of wild-type venules, no rolling leukocytes are seen in the mesentery of the P-selectin–deficient mice.[15] This shows that P-selectin is indispensable in mediating leukocyte rolling in the otherwise unstimulated blood vessels. Leukocyte rolling is thought to be the first required step in leukocyte recruitment to sites of inflammation.[1,16-18] Indeed, early after induction of an inflammatory response through injection of thioglycollate into the peritoneum of the P-selectin–deficient animals, there is very little neutrophil recruitment into the peritoneum in comparison with that seen in wild-type animals. In contrast, four hours after the thioglycollate injection, the rate of neutrophil recruitment in the knock-out animals is comparable with that in the wild type.[15] One of the questions we address in the current study is whether, at this later time when neutrophils appear to extravasate normally, the rolling of leukocytes is also restored in the P-selectin–deficient mesentery.

Finally, in our initial characterization of the P-selectin–deficient animals,[15] we have examined only recruitment of neutrophils early after the onset of inflammation. Although neutrophils are responsible for fighting infection during the early stages of the inflammatory response, if the initial insult

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is arrested, the infiltrate becomes primarily mononuclear in nature. In this report, we examine whether P-selectin also plays a role in the recruitment of monocytes/macrophages in these later stages of the inflammatory response.

MATERIALS AND METHODS

Animals

Mice were maintained and bred in a conventional animal facility at Center for Cancer Research, Massachusetts Institute of Technology or Tufts-New England Medical Center or Center for Blood Research, Harvard Medical School. Animals were housed in accordance with the provisions of the Guide for the Care and Use of Laboratory Animals. All experimental procedures performed on animals were reviewed and approved by the Animal Care and Use Committees of Tufts-New England Medical Center, Boston, MA and Center for Blood Research, Boston, MA. These institutions are accredited by the American Association for Accreditation of Laboratory Animal Care.

Blood/Tissue Collection and Staining

Blood was collected from retro-orbital venous plexus sampling. The hematocrit was measured with an autocrit centrifuge (Clay Adams, Parsippany, NJ). Reticulocytes were stained for 10 minutes with methylene blue using a 1:1 dilution of blood with a 5 g/L stock solution. Smears were made and the number of reticulocyte was assessed by counting 1,000 red blood cells (RBCs). Spleens from 12 wild-type and 13 P-selectin–deficient mice were collected, fixed in formalin, and paraffin embedded. Sections were stained for iron based on the Prussian Blue reaction according to manufacturer's instructions (Sigma, St Louis, MO).

Flow Cytometry

Leukocytes in whole blood were stained for 1 hour on ice with direct fluorescent conjugates diluted 1:50 for Mac-1 (Boehringer-Mannheim, Indianapolis, IN), CD4, CD8, IgM, and natural killer (NK) cells (NK1.1) were from Pharmingen, San Diego, CA. Erythrocytes were lysed by adding nine volumes of lysing solution (Becton Dickinson, San Jose, CA) diluted 1:10. The cells were washed with phosphate-buffered saline (PBS) after 10 minutes incubation at room temperature. Leukocyte subsets were analyzed on a FACSTAR Plus flow cytometer (Becton Dickinson). Gate was set to include all leukocyte subsets. The values for IgM-positive cells were lower than expected for normal mouse. This is possibly caused by the formaldehyde present in the lysis solution. Cells from organs (thymus, spleen, lymph node) were stained as above after collection by compressing tissue through 60 μm wire mesh and washing with ice-cold PBS with 0.2% bovine serum albumin (BSA). All negative controls were stained with conjugated rat-antimouse igG.

Demargination

To examine the effect of P-selectin deficiency on the margination of neutrophils, blood samples were taken from wild-type and mutant mice 24 hours before intraperitoneal injection of 0.25 mg/kg epinephrine (BIO/DATA, Horsham, PA). To avoid effects on blood pressure, anesthesia was avoided and blood samples were taken via tail vein 35 minutes after injection of epinephrine. Total white blood cells in preepinephrine and postepinephrine blood samples were determined by Coulter counter and differential counts were determined on Wright-Giemsa–stained smears.

Neutrophil Clearance

Human blood was collected in one-tenth volume of 3.8% citrate, 0.15 mol/L NaCl and sedimented (1g) in 1% final concentration Dextran (molecular weight, 100,000) for 30 minutes at room temperature. The supernatant was washed twice with PBS, resuspended to the original volume and layered over Ficoll-Hypaque (Pharmacia, Piscataway, NJ) then centrifuged at 400*g* for 30 minutes. Mononuclear cells at the interface were carefully removed and discarded and the pellet was washed twice. The remaining RBCs were lysed by brief exposure to hypotonic solution and made isotonic by addition of excess PBS. The neutrophils (>95%) were assessed by staining with Wright-Giemsa (Baxter, Glendale, CA) and were >95% viable by trypan blue exclusion. The cells were then labeled with ^{51}Chromium for 20 minutes at 37°C. Mice were injected via the tail vein with 2×10^6 neutrophils.

In Vitro Lymphocyte Binding Assay

Lymphocyte adherence to high endothelial venules (HEVs) in vitro was assayed by the method of Butcher et al,[19] a modification of the assay originally described by Stamper and Woodruff.[20] Unfixed frozen sections (5 μm) of peripheral and mesenteric lymph nodes of wild-type and P-selectin–deficient mice were overlayed with 1×10^6 lymphocytes/100 μL and incubated at 4°C. Lymphocytes were derived from peripheral and mesenteric lymph nodes of female C57BL/KA-Thy1.1 mice. During this assay, lymph node sections were rotated for 30 minutes. Upon completion of the assay, unbound cells were decanted, and sections were fixed in PBS containing 2.5% glutraldehyde for 1 hour. Cell binding to HEV was assessed microscopically under dark field illumination and the mean number of bound cells per HEV was determined.

Immunohistochemistry

Cryostat sections of lymph nodes (5-μm thickness) were allowed to dry for 15 minutes before fixation in acetone (2 minutes) after which they air dried for another 15 minutes. The sections were incubated with various rat-antimouse antibodies at saturating concentrations in PBS containing 0.1% BSA (PBS/BSA) for 45 minutes. After washing thoroughly in PBS, sections were incubated for 30 minutes with 1:150 dilution of peroxidase-conjugated goat-antirat IgG/IgM (Jackson Immunoresearch, San Francisco, CA) in PBS/BSA containing 5% normal mouse serum. After washing in PBS, the peroxidase activity was detected with 3-amino-9-ethylcarbozole (AEC, Biomeda, Foster City, CA).

Neutrophil/Platelet Rosetting Assay

Blood from wild-type and mutant mice was collected in EDTA and platelets were collected by filtering platelet-rich plasma through a Sepharose 2B (Pharmacia, Piscataway, NJ) column equilibrated with 25 mmol/L PIPES, 137 mmol/L NaCl, 4 mmol/L KCl and 0.1% wt/vol glucose at pH 6.8. For rosetting, platelets were activated with 0.15 U/mL thrombin for 10 minutes at room temperature. The neutrophils were collected as described above for human blood from pooled mouse blood from both wild-type (~25 mice/experiment) and mutant mice (>20 mice/experiment). However, the supernatant after RBC sedimentation was layered over lympholyte M (d, 1.087) (Cedar Lane Labs, Hornby Ont, Canada) and centrifuged for 30 minutes at 1,000*g*. Twenty microliters of platelets at 10^7/mL were mixed with 20 μL neutrophils at 2×10^7/mL and incubated for 20 minutes before observation. A neutrophil was considered positive for rosetting when there were two or more bound platelets on the surface.

Thioglycollate-Induced Peritonitis

For experimentally induced chronic peritonitis, mice were injected with 1 mL thioglycollate (Sigma) made according to manufacturer's instructions. After 24 and 48 hours, blood samples were taken, mice

were killed and 10 mL PBS containing 0.1% BSA, 0.5 mmol/L EDTA, and 0.1 U/mL heparin were used to lavage the peritoneum. Total cells were quantitated by Coulter counter and cytospin preparations of the cells were stained with Wright-Giemsa and differentially counted. For cytospin preparations, 200 cells were counted independently (coded slides) by two investigators (400 total). Similarly, 100 cells were counted independently (200 total) for blood smears.

For accurate quantitation of resident macrophages (unstimulated), peritoneal lavage was performed on untreated mice and cytospin samples were stained with a monoclonal antibody, F4/80, specific for monocyte/macrophages, secondarily labeled with biotinylated antimouse antibody followed by streptavidin-peroxidase conjugate and appropriate chromogen reaction according to manufacturer's instructions (Vector, Burlingame, CA). The slides were counterstained with hematoxylin and differential counts were performed and the total number of resident macrophages was calculated.

Intravital Microscopy

Mice were given 1 mL thioglycollate 3.5 hours before surgery, then anesthetized with 2.5% tribromoethanol 0.15 mL/10 gm and the mesentery was exteriorized through an abdominal incision. A 25- to 35-μm venule was video-recorded using a Zeiss IM35 inverted microscope (objective × 32, 0.4 numerical aperture) connected to a SVHS video recorder (Panasonic, Tokyo, Japan) using a CCD video camera (Hamamatsu Photonic Systems, Tokyo, Japan). Exposed tissue was periodically moistened with PBS warmed to 37 C. Venules were filmed for 30 minutes and the average number of rolling leukocytes determined by taking a minimum of 8 one-minute counts. Velocity was determined by the time required for a leukocyte to travel a defined distance (μm) along the vessel. A minimum of 10 measurements were taken for wild-type animals and eight measurements for P-selectin–deficient mice with the exception of one mutant animal where only one measurement could be obtained. For baseline (without stimulation) velocity measurements from wild-type mice, eight measurements were taken. Leukocytes that remained stationary for 30 seconds or more were considered adherent.

RESULTS

Characterization and Distribution of Blood Cells in the P-Selectin–Deficient Animals

BM. Smears were made of BM from wild-type and P-selectin–deficient mice and erythroid and myeloid precursors were differentially quantitated (Table 1). The myeloid to erythroid ratio was 1.9 ± 0.2 for both strains of mice and there were no distinctive differences in lineage differentiation. Megakaryocyte numbers appeared somewhat higher in mutant marrow smears. Further comparison of megakaryocyte numbers by flow cytometry using von Willebrand factor as a marker showed that the difference (wild type, 0.35% ± .06%; mutant, 0.44% ± .06%, where n = 10) was not statistically significant (P = .297).

Table 1. BM Differential Count

	Wild Type (% ± SD) (n = 6)	Mutant (% ± SD) (n = 6)
Myeloblasts	0.9 ± 0.4	0.8 ± 0.2
Promyelocytes	2.3 ± 0.4	2.1 ± 0.7
Myelocytes	4.6 ± 0.7	3.1 ± 0.6
Metamyelocytes	4.3 ± 0.8	3.7 ± 0.8
Bands	14.8 ± 0.6	15.4 ± 2.3
Neutrophils	32.5 ± 2.1	35.5 ± 2.0
Monocytes	0.1 ± 0.1	0.3 ± 0.2
Lymphocytes	8.6 ± 1.3	6.1 ± 0.8
Plasma cells	0.2 ± 0.1	0.1 ± 0.1
Pronormoblasts	0.2 ± 0.1	0.3 ± 0.3
Basophilic normoblasts	5.8 ± 1.5	3.5 ± 0.9
Polychromatophilic normoblasts	24.9 ± 3.4	29.0 ± 2.3
Orthochromic normoblasts	0.3 ± 0.2	0.4 ± 0.3
Myeloid:erythroid ratio	1.9 ± 0.2	1.9 ± 0.2

Comparison of BM precursors in wild-type and P-selectin–deficient mice. Marrow was collected from the femur, smeared onto glass slides and stained in separate steps with Wright's and Giemsa.

Table 2. Cytofluorimetric Analysis of Leukocyte Subsets

Tissue of Origin	Antigen	Wild Type (%)	Mutant (%)
Blood	CD4	18.2 ± 2.5	14.9 ± 2.0
	CD8	8.0 ± 1.4	6.9 ± 1.7
	Mac 1	23.1 ± 6.9	51.7 ± 3.3
	IgM	5.6 ± 3.1	5.2 ± 3.5
	NK	6.1 ± 1.3	5.6 ± 0.7
Lymph node	CD4	51.9 ± 5.1	48.1 ± 4.5
	CD8	30.7 ± 1.9	31.1 ± 1.0
	CD3	76.1 ± 5.3	71.5 ± 2.6
	IgM	12.5 ± 4.7	13.2 ± 4.4
Spleen	CD4	17.5 ± 1.2	18.2 ± 1.3
	CD8	9.4 ± 0.8	7.1 ± 2.2
	Mac 1	15.6 ± 3.3	18.3 ± 6.7
	IgM	46.7 ± 9.0	38.0 ± 5.8
Thymus	CD4	90.3 ± 2.6	91.9 ± 2.2
	CD8	80.1 ± 1.1	79.0 ± 3.7
	CD4 CD8	78.5 ± 0.7	78.9 ± 6.6
	CD3	7.7 ± 0.6	7.9 ± 0.7
	Thy 1.2	92.6 ± 2.3	97.5 ± 1.6

Cells were stained for the indicated antigens using fluorescent-conjugated antimouse antibodies. Data include the mean ± SEM from three separate experiments and are presented as percent of total displaying positive fluorescence. All the percentages in the wild-type mice conform to reference values except for that of the IgM-positive cells in the blood, which is lower (see Materials and Methods).

Blood cells. Leukocytes from wild-type or P-selectin–deficient mice were collected and labeled for markers indicated in Table 2. Comparable cell counts were obtained from the spleen, lymph nodes, and thymus from both types of mice. No significant differences were observed between wild-type and P-selectin–deficient mice for the markers selected with the exception of Mac-1–positive cells in the blood where the difference reflects the higher numbers of neutrophils in the mice lacking P-selectin.

We have also confirmed that P-selectin is the major ligand for neutrophil binding to the activated platelets.[21,22] Resting or activated platelets from wild-type mice and mice deficient for P-selectin were incubated with neutrophils isolated either from wild-type or mutant mice. Normal mouse platelets activated with thrombin efficiently bound the neutrophils, whereas platelets collected from mice deficient for P-selectin failed to rosette with neutrophils from either strain. Resting platelets from both genotypes likewise failed to bind significantly to the neutrophils (not shown).

Venules in the lymph nodes are known to express P-selectin.[7] Therefore, we examined adhesion of wild-type mouse lymphocytes to high endothelial venules in lymph node sections obtained from wild-type and P-selectin–defi-

cient mice. The binding of lymphocytes to high endothelial venules in peripheral and mesenteric lymph nodes of homozygous mutant mice was comparable with the binding to wild-type nodes (Table 3). Staining of peripheral lymph nodes using monoclonal antibody MECA-325, and of mesenteric lymph nodes with MECA-325 and MECA-367, showed the presence of high walled vessels in both genotypes (not shown).

We found no difference in hematocrit for wild-type (36.8 ± 2.5) and P-selectin–deficient (35.8 ± 3.6) mice after comparison of five animals. Similarly, splenic hemosiderin, although variable between animals, was present at equivalent levels for both genotypes. Reticulocyte counts were also similar (1.8 ± 0.3% v 2.0 ± 0.4%) for the P-selectin–deficient and wild-type mice, respectively. These data combined with the normal number of erythroid precursors in the BM suggest that P-selectin is not essential for RBC generation nor removal from circulation.

Demargination of neutrophils. Because there are no obvious alterations in neutrophil precursor numbers in the BM, we reasoned that the higher levels of circulating neutrophils in the mutant animals could be caused by delayed clearance or to an effect on the marginated fraction of blood neutrophils. "Marginated" neutrophils are thought to reside predominantly in lung and spleen and are not detected on routine blood sampling. However, they are a readily accessible pool of neutrophils that are rapidly mobilized into the general circulation in response to epinephrine or cortisol.

To test whether P-selectin–deficient mice and wild-type animals have a comparable marginated pool of neutrophils, we collected blood samples before and after injection of epinephrine. Neutrophil numbers from wild-type mice increased about fourfold from 4.7×10^5 /mL to 17.7×10^5 /mL (Fig 1). P-selectin–deficient mice also responded to the epinephrine with an approximately twofold increase in circulating neutrophils from 15.8×10^5/mL to 28.0×10^5 /mL. The increase in both types of mice is statistically significant. Although the ratios of response for wild-type mice (4×) relative to P-selectin–deficient mice (2×) are different, the absolute numbers of neutrophils released into circulation in response to epinephrine are comparable (wild-type, 13×10^5; mutant, 12.2×10^5). This suggests that the higher peripheral neutrophil count in the mutant mice is not caused by a reduction in the marginated pool, wherever that pool resides.

Table 3. Lymphocyte Adherence to HEV

	No. of HEVs	No. of Bound Cells	Cells:HEV
PLN wild type (female)	14	210	15.0
PLN wild type (male)	14	268	19.1
PLN mutant (female)	17	239	14.1
PLN mutant (male)	15	233	15.5

In vitro binding of lymphocytes to HEVs in wild-type and homozygous mutant PLNs. Frozen sections were incubated with lymphocytes and rinsed, and the numbers of adherent lymphocytes were counted under the microscope.

Abbreviation: PLNs, peripheral lymph nodes.

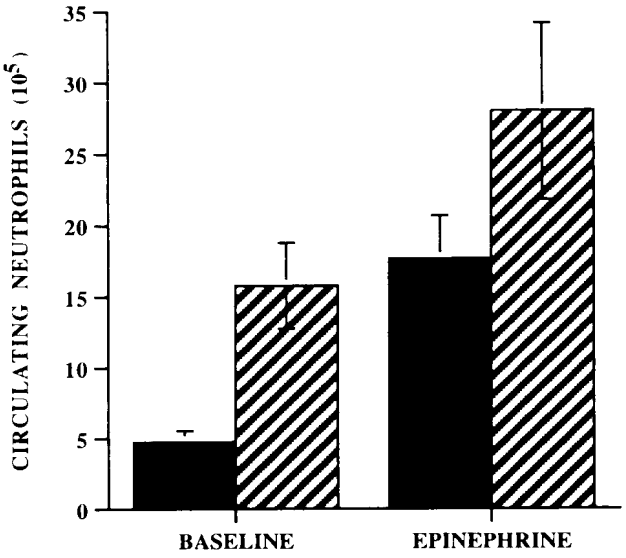

Fig 1. Epinephrine-induced demargination in wild-type and P-selectin–deficient mice. Blood samples were collected from mice 24 hours before (baseline) and 35 minutes after (epinephrine) intravenous injection of 0.25 mg/kg epinephrine. Total counts were determined by Coulter counter and differential counts were determined on stained blood smears. The neutrophilia in response to epinephrine was statistically different from baseline for wild-type (■) ($P < .01$; n = 7) and for P-selectin–deficient (▨) ($P < .05$; n = 8) mice. Data were compared using the Student's *t* statistic for paired data. The bar graph is presented as mean ± SEM.

Neutrophil clearance. A delay in neutrophil clearance in the P-selectin–deficient mice could account for the basal neutrophilia in these animals. To assess this possibility, ^{51}Chromium-labeled human neutrophils were injected into the tail vein of normal and homozygous mutant animals. Organs (lung, liver, spleen, kidney) and blood were collected at various time points after the intravenous injection. The amount of radioactivity found in the organs was similar in the wild-type and P-selectin–deficient mice (not shown). Interestingly, mean counts were higher in the spleen of wild-type mice for the 40-, 75-, and 135-minute time points, but this difference was not statistically significant. The presence of ^{51}Chromium in these organs affirmed generalized circulation of the human neutrophils. There was no statistically significant difference in counts from blood samples collected immediately (5 to 15 minutes) after injection of neutrophils. However blood samples collected at 40, 75, and 135 minutes after injection exhibited a striking difference in radioactivity. Radiolabeled neutrophil counts were significantly higher in the mutant animals than in the wild-type at all three time points. The ratio of counts recovered from mutant blood to counts from wild-type blood were the highest at 75 minutes after injection (Table 4). At 5.5 hours after injection, the counts dropped to low levels in both genotypes and a difference was no longer detected.

Circulating neutrophils may compete for a limited number of binding sites when targeted for removal from the blood stream. The P-selectin–deficient mice, having higher numbers of circulating neutrophils, would have a fundamental

Table 4. Comparison of Neutrophil Clearance Between Wild-Type and P-Selectin–Deficient Mice

Time (min)	No. of Animals (wild type:mutant)	Mean cpm: Mutant/Mean cpm: Wild Type	P Value*
5-15	4:4	0.97	.88
40	9:7	1.33	.02
75	13:13	1.82	.001
135	6:6	1.49	.004
330	3:4	0.96	.75

* cpm were normalized for counts injected, and counts obtained from wild-type and mutant mice were compared using Student *t* statistics for unpaired data.

disadvantage for clearance after injection of heterologous neutrophils if the total circulating neutrophils were to exceed this finite capacity for binding. To examine the influence of circulating neutrophils on the clearance of injected neutrophils, we repeated the procedure and determined radioactivity in blood, reflecting injected cells only, and peripheral blood neutrophil numbers for each animal. Regression analysis was generated separately for each genotype to determine if there is a correlation between peripheral neutrophil numbers, and counts acquired from injected radiolabeled cells. The determination coefficients for wild-type (r^2 = .022) and P-selectin–deficient mice (r^2 = .191) were low indicating an insignificant correlation to peripheral neutrophil counts with respect to the numbers of labeled neutrophils added to the circulation. These results suggest that increased neutrophil counts in the P-selectin–deficient mice are likely the consequence of retarded clearance from the blood stream.

Experimentally Induced Peritonitis

In our previous study[15] we reported that mobilization of neutrophils during the first two hours after induction of peritonitis was severely compromised in P-selectin–deficient mice. In the present study, we wished to extend this analysis to later times and also to examine the recruitment of monocytes that exhibit a more delayed course in response to tissue injury.

Leukocyte extravasation. P-selectin–deficient and wild-type mice were injected with 1 mL thioglycollate in the peritoneum and killed 24 or 48 hours later. Although peritoneal neutrophil counts were almost twofold higher in the P-selectin–deficient mice at 24 hours, this difference was not statistically significant (P = .057). Neutrophil counts in the inflamed peritoneum were indistinguishable between mutant and wild-type mice at 48 hours (Table 5). Similarly, there was no difference in eosinophils in the peritoneum at 24 (wild-type, 3.8×10^5; mutant, 4.9×10^5) or 48 (wild-type, 7.4×10^5; mutant, 5.9×10^5) hours.

Table 5. Total and Differential Counts of Neutrophils in Thioglycollate-Induced Peritonitis

Time (hrs)	Total Neutrophils (%)	
	Wild Type	Mutant
1.25*	$0.48 \cdot 10^6$ (6.6)	$0.017 \cdot 10^6$ (0.1)
2.25*	$3.2 \cdot 10^6$ (32.9)	$0.5 \cdot 10^6$ (9.8)
4.0*	$10.9 \cdot 10^6$ (60.0)	$5.2 \cdot 10^6$ (46.5)
24	$1.59 \cdot 10^6$ (27.4)	$3.15 \cdot 10^6$ (33.4)
48	$1.15 \cdot 10^6$ (13.8)	$0.72 \cdot 10^6$ (10.2)

* Data from Mayadas et al.[15]

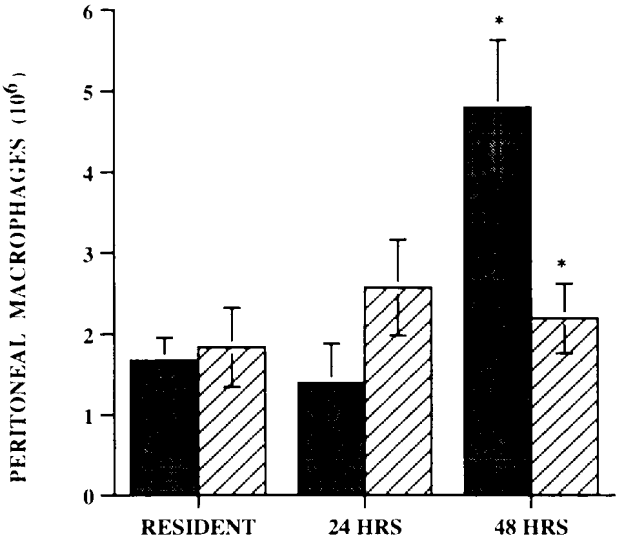

Fig 2. Macrophage recruitment in thioglycollate-induced peritonitis. Thioglycollate was administered intraperitoneally 24 or 48 hours before collection of peritoneal infiltrate. Total cell counts were determined using a Coulter counter and samples were differentially quantitated on Wright-Giemsa stained cytospin preparations. Differential counts for resident macrophages (resident) were performed on cytopsin preparations labeled with a monocyte/macrophage specific antibody (F480). There was no statistical difference between resident macrophages (n = 3) and macrophages recovered at 24 hours (n = 8) for either genotype. In contrast, the wild-type (■) mice exhibited a significant increase in peritoneal macrophages at 48 hours, whereas the P-selectin–deficient (▨) mice fail to show any increase after thioglycollate. The asterisk denotes statistical difference ($P < .02$) between wild-type and homozygous mutant mice at 48 hours where n = 9. Data were compared using the Student's *t* statistic for unpaired data. The bar graph is presented as mean ± SEM.

By 1 and 2 days postinjection, neutrophils represent a less significant percentage of the total peritoneal cells than at early times and are being replaced by macrophages, which differentiate from peripheral blood monocytes extravasating through mesenteric venules. As shown in Fig 2, the numbers of macrophages at 24 hours are not significantly different from the starting population of resident macrophages. Furthermore, there is no significant difference between mutant and wild-type mice. However, by 48 hours, the number of macrophages in the wild-type mice had more than tripled, whereas that in the mutant mice remained unchanged. There was no significant difference in numbers of circulating monocytes (not shown).

Therefore, even during the second day of inflammation, P-selectin continues to play a role in leukocyte recruitment. Because the numbers of neutrophils at 1 and 2 days are not significantly different between the mutant and wild-type mouse strains, it seems unlikely that the deficit in monocyte

Table 6. Behavior of Leukocytes Under Baseline and Inflammatory (Thioglycollate) Conditions

	Wild Type	Mutant	*P* Value
Baseline rolling (cells/min)* (n = 8)	10.5 ± 2.5	0.05	.001
Baseline velocity (μm/s) (n = 5)	26.1 ± 1.8	NA	NA
Thioglycollate rolling (cells/min) (n = 6)	48.9 ± 12.6	1.4 ± 0.4	.004
Thioglycollate velocity (μm/s) (n = 6)	23.7 ± 2.4	7.1 ± 0.6	.001
Thioglycollate adherent (cells/100 μm) (n = 6)	3.3 ± 0.6	3.6 ± 0.9	.765

The number of visible cells (diffractile spheres) crossing a given plane perpendicular to the vessel axis were counted (rolling). Velocity was determined as the time required to travel a defined distance along the vessel. Cells were considered adherent if they remained stationary for 30 seconds or more. Data were compared using the Student's *t* statistic for unpaired data. The data are presented as mean ± SEM.

* Data from Mayadas et al[15] for comparison.

recruitment is a secondary consequence of early deficits in neutrophil extravasation. Rather, it appears that P-selectin, which may be expressed in chronic fashion in the blood vessels of the inflamed peritoneum, plays a role in monocyte extravasation.

Leukocyte rolling/intravital microscopy. Because the rate of recruitment of neutrophils at 4 hours after thioglycollate injection was similar in both strains of mice, we expected to observe leukocyte rolling also in the P-selectin–deficient mice. This could be a consequence of the induction of E-selectin. Therefore, we performed intravital microscopy at 3.5 to 4.5 hours postthioglycollate injection. To our surprise, we continued to detect a marked difference in the rolling behavior of leukocytes in P-selectin–deficient and wild-type mice (Table 6). Although rolling was now also observed in the mutant mice, the numbers of rolling cells were greatly reduced (≈3% of wild-type) and cell movement was different from that seen in the wild-type mice. The rolling cells in inflamed vessels of the P-selectin–deficient mice moved threefold more slowly than in the wild-type mice (Table 6) and were characterized by a very retarded motion as if crawling along the vessel wall. There was more variation in velocity of the leukocytes in the wild-type mice. With both genetic backgrounds, after thioglycollate injection, more cells were adherent to the vessel wall than under baseline conditions. On the average, there were 3 to 4 adherent leukocytes over a venular length of 100 μm in both the wild-type and P-selectin–deficient mice (Table 6). Consequently, although the rate of extravasation of neutrophils at 4 hours is similar in wild-type and mutant mice, this similarity is not reflected in the behavior of rolling leukocytes in the mesenteric venules. Because the numbers of rolling cells are much lower in the mutant mice, the conversion of rollers to adherent and/or extravasated cells appears to be more efficient than in the wild-type mice.

DISCUSSION

Previously we had shown profound changes in white blood cell (WBC) behavior in mice lacking P-selectin on platelets and endothelium.[15] In this report, we evaluated dynamics of WBC subsets including neutrophils, lymphocytes, and monocytes. No defects were observed in the lymphocyte subpopulations we tested, suggesting that lymphocyte differentiation and homing is not excessively perturbed (Table 2). This is supported by results obtained in the Stamper-Woodruff assay examining lymphocyte adhesion to lymph nodes obtained from P-selectin–deficient mice (Table 3). Lymphocytes adhere normally to lymph-node sections from P-selectin–deficient animals suggesting, in addition, that under these conditions L-selectin on lymphocytes does not present a functionally important ligand for P-selectin within peripheral lymph node venules. Because the presence of L-selectin is crucial for lymphocyte binding to the peripheral lymph nodes,[24,25] an L-selectin ligand distinct from P-selectin, perhaps GlyCAM-1[26] or CD34,[27] must be used. In contrast with L-selectin–deficient mice that have mostly flat endothelium in the peripheral lymph nodes,[24] the P-selectin deficient animals' endothelium had a normal appearance.

Both strains of mice have similar myeloid:erythroid ratios in the BM that also support the conclusion that there are no major alterations in leukocyte maturation. Equal numbers of myeloid precursors and mature neutrophils in the BM of wild-type and P-selectin deficient mice (Table 1) indicate that the elevated numbers of blood neutrophils in P-selectin–deficient mice are primarily a result of an alteration that occurs in the blood vessels. The mechanisms underlying the control of neutrophil margination and the regulation of stimulated release of neutrophils into general circulation (demargination) are not well understood.[28] We wondered whether the lack of P-selectin could influence the margination of neutrophils, thereby effectively reducing the pool of marginating neutrophils and increasing the circulating pool. The wild-type mice responded to an injection of epinephrine with a fourfold increase in circulating neutrophils, whereas the mutant strain exhibited a twofold elevation (Fig 1). However, as indicated in Results, the total pool of neutrophils released into circulation was almost identical in both strains. Possibly, the margination event is independent of adhesion molecules and is dependent on release of neutrophils from spleen and lung upon physical contraction of these organs. Nevertheless, these results indicate that the total marginated pool in both wild-type and P-selectin–deficient mice is comparable and support the theory that margination is not merely a reflection of a constitutive rolling pool of neutrophils.

The presence of a normal marginated pool of neutrophils and the normal appearance of the BM in the P-selectin deficient animals suggest that the observed neutrophilia may be caused by a delayed clearance of neutrophils from circulation. The half-life of neutrophils in the circulation in humans is 6 to 9 hours; thus, the turnover of this population is relatively rapid.[29] Our studies on neutrophil clearance showed a reduction in radiolabeled cells in wild-type mice, whereas their removal in the mutant animals was delayed over the first 2 to 3 hours. (Table 4). It is clear that other mechanisms for neutrophil clearance exist because numbers were equivalent by 5 hours after intravenous injection of labeled cells. These results suggest that P-selectin is important for the normal turnover of neutrophils in circulation. It is interesting

to note that mice deficient for L-selectin do not have an elevation in neutrophil numbers, but present a prominent defect in neutrophil recruitment to inflamed tissue.[23] This suggests that recruitment at sites of inflammation and clearance of senescent neutrophils do not use the same adhesion mechanisms. It is intriguing that intercellular adhesion molecule-1 (ICAM-1)–deficient mice have even higher (fourfold) elevation in neutrophils[30,31] than do P-selectin–deficient mice, and leukocyte-adhesion deficiency (LAD) I patients, who lack β_2 integrin, have a 5- to 20-fold increase.[32] The marginated pool of neutrophils is unaffected in LAD I patients, but there is delayed clearance of neutrophils (approximately twofold),[33] similar to what we observe in the P-selectin–deficient mice. Because the integrin $\alpha_M\beta_2$ on neutrophils binds ICAM-1, possibly the ICAM-1 deficiency or the β_2 deficiency does not allow firm adhesion to endothelium and, therefore, removal of neutrophils from circulation is impaired. This adhesive mechanism remains intact in P-selectin–deficient mice and may explain why the injected neutrophils are cleared from circulation at 5 hours and why the neutrophilia is less pronounced.

Larsen et al[21] had previously shown that platelet/neutrophil rosetting could be inhibited with antibodies to P-selectin. Using platelets recovered from P-selectin–deficient mice, we have confirmed that P-selectin is indeed the major adhesion molecule contributing to this event. This was true for neutrophils isolated from both wild-type mice and P-selectin–deficient mice. It is notable that activated platelets from wild-type mice would efficiently rosette with neutrophils recovered from mice deficient for P-selectin. This showed that it is possible for neutrophils from the mutant animals to bind to P-selectin and, therefore, by extension, they are capable of rolling on activated endothelium expressing P-selectin. This suggests that the defect in rolling exhibited in the mutant animals is solely caused by deficiency of P-selectin on endothelium and not by a neutrophil defect.

Our previous studies show that a lack of P-selectin affects neutrophil recruitment during the initial 2 hours of experimentally induced peritonitis. Therefore, it is apparent that P-selectin plays a major role in cell recruitment in acute inflammation where neutrophils predominate. Similarly, studies using a soluble L-selectin IgG chimera[34] and recent data with L-selectin–deficient mice[24] indicate that L-selectin is important in acute thioglycollate-induced peritonitis. At 4 hours, in both P-selectin– and L-selectin–deficient mice, the total peritoneal neutrophils begin to approach those recovered from wild-type mice and at 24 and 48 hours, we observe no significant differences in peritoneal neutrophil counts.

Perhaps the most striking result in the peritonitis model was the failure to recruit significant numbers of macrophages into the peritoneum of mice lacking P-selectin (Fig 2). In principle, this could be a consequence of fewer neutrophils at early times in the peritoneum and, therefore, reduced chemoattractant activity for macrophages. However, the neutrophil numbers in the mutant mice at 24 hours indicate that total neutrophil recruitment, although delayed, is not significantly impaired. It is possible that the neutrophil contributes to the recruitment of the macrophage by receiving a signal upon P-selectin binding that induces production of a substance involved in macrophage recruitment. If such process were involved, the P-selectin deficient mouse would still have the low observed macrophage recruitment.

The inflammatory dynamics at 48 hours, when macrophages are engaged, therefore appears to shift back to one that is P-selectin dependent. The role of P-selectin in monocyte binding is supported by results from other laboratories. Monocyte adhesion to inflamed venules was examined in a modified Stamper-Woodruff frozen-section binding assay using synovial biopsy samples from patients with rheumatoid arthritis. P-selectin was shown to be the principal mediator of monocyte adhesion to synovial venules as antibodies to P-selectin could inhibit binding greater than 90%. Control sections of normal foreskin and placenta exhibited minimal to no P-selectin–mediated adhesion.[35] Moreover, increased luminal expression of P-selectin has been detected overlying human atherosclerotic plaques of all active developmental stages including the fatty streak, whereas no expression is observed overlying inactive fibrous lesions or normal arterial endothelium.[36] Stronger expression was commonly observed where there was concentrated macrophage infiltration. In a separate study, the expression of adhesion molecules was examined in sections of thyroid glands from patients with an autoimmune thyroiditis (Graves' disease). The investigators found a positive correlation between expression of P-selectin on endothelial cells and mononuclear cell infiltration in affected thyroid glands.[37] These observations combined with ours suggest that P-selectin may play a role in the recruitment of macrophages to chronically inflamed tissue. This is consistent with data indicating that P-selectin cycles back to the Weibel-Palade body and from there can be reexpressed on the surface of endothelium.[14] Moreover, inflammatory cytokines also upregulate surface expression of P-selectin while inducing E-selectin.[15]

To our surprise, we found that rolling of leukocytes was still altered in the mutant mice at 4 hours after thioglycollate injection (Table 6). We selected the 4-hour time point because E-selectin is likely to be expressed[11] and P-selectin–deficient mice apparently regain the capacity to recruit neutrophils.[15] Because of the large difference in the rolling leukocyte numbers (48.9:1.4) in the two genotypes, and no difference in the rate of extravasation,[15] it appears that the number of rolling leukocytes is not a good predictor of the extent of leukocyte extravasation. The reduced rolling velocity of leukocytes was a conspicuous feature in the P-selectin–deficient mice. This is likely because of a stronger adhesive interaction that mediates leukocyte rolling and prevails in the P-selectin deficient animals. Interestingly, Lawrence and Springer[38] recently showed that under flow conditions in vitro, the adhesion of neutrophils to E-selectin is stronger than to P-selectin; consistent with our data in vivo, leukocyte rolling velocity was slower and less variable when rolling on E-selectin than on P-selectin. Moreover, the apparent increased efficiency of conversion to firm adhesion with the low level of rolling we observed in the mutant mice could be explained by the observation of Lo et al[39] that rolling on E-selectin can activate neutrophils and increase adhesive potential of CD11b/CD18 (α_M/β_2). Possibly, prominent P-

selectin expression in the wild-type animals is masking E-selectin and the resulting rapid rolling on P-selectin produces less efficient activation. It is possible that the rolling population in normal mice comprises two pools: one large and fast-rolling, and perhaps converting poorly or not at all to adherence, and a second, smaller pool with slower rolling velocity and higher conversion to adherence. If this were the case, then one would suppose that the first pool is missing in the P-selectin–deficient mice, whereas the second pool persists and accounts for the extravasation of neutrophils in the mutant mice. In this model, the first, fast-rolling pool is P-selectin-dependent and the second, slower rolling pool relies on some other molecule(s). Whatever the case, it is clear, both from the reduction in leukocyte rolling at four hours and from the deficit in macrophage recruitment at 1 to 2 days in the P-selectin–deficient mice, that P-selectin might significantly influence the course of various chronic inflammatory disorders and is not solely involved in early, acute phases of inflammation.

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EXHIBIT

F

Reduced Recruitment of Inflammatory Cells in a Contact Hypersensitivity Response in P-Selectin–deficient Mice

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Summary

The inflammatory response at sites of contact hypersensitivity induced by oxazolone was examined in the ears of P-selectin–deficient and wild-type mice. Accumulation of $CD4^+$ T lymphocytes, monocytes, and neutrophils was reduced significantly in the mutant mice, as well as mast cell degranulation. In contrast, there was no significant difference in vascular permeability or edema between the two genotypes. The results demonstrate a role for P-selectin in recruitment of $CD4^+$ T lymphocytes and show that P-selectin plays a role in long-term inflammation as well as in acute responses.

Leukocyte recruitment to sites of inflammation involves several steps: Leukocytes initially tether and roll on the activated endothelium; they subsequently become activated and bind firmly through leukocyte integrins; and they emigrate into surrounding tissues. Leukocyte rolling is a weak, reversible interaction mediated by the selectins, which appear to have overlapping functions (1–3). In the initial phase, P-selectin is the primary mediator of leukocyte rolling (4). L-selectin that is present on the leukocytes is also involved in rolling but most likely in a later time frame (5). E-selectin is expressed on activated endothelium hours after the onset of inflammation and may participate in rolling, as demonstrated recently in vitro (6).

The role of P-selectin in recruitment of neutrophils and monocytes has been studied in chemical peritonitis, acute lung injury, and ischemia reperfusion (3). However, little is known about its function in lymphocyte-mediated chronic inflammation in models such as contact hypersensitivity (CH), a form of delayed type hypersensitivity. In this cutaneous inflammatory model, the sensitizing agent penetrates the skin and binds Langerhans' dendritic cells. These cells act as antigen-presenting cells and activate the T lymphocytes in the local lymph node to undergo proliferation (7). Upon subsequent challenge with the same antigen, the primed $CD4^+$ T lymphocytes, assisted by a subset of $CD8^+$ lymphocytes (7, 8), mount a CH response at the site of challenge by secreting several cytokines lymphokines (7, 9). Some of these cytokines activate the endothelium to express adhesion molecules or their ligands (3), which help to recruit monocytes, more lymphocytes, and neutrophils to the site of challenge. The recruited macrophages further amplify the reaction by secreting TNF-α and IL-1 (10), which increase the expression of P-selectin, E-selectin, the ligand for L-selectin, intracellular adhesion molecule 1, and vascular cell adhesion molecule on the surface of endothelial cells (3). Histamine and serotonin released by mast cells and possibly other cells such as platelets (11, 12) may be important in the early phase of the reaction. Histamine and serotonin cause release of P-selectin from the Weibel-Palade bodies (13, 14), and this also induces leukocyte rolling in vivo (15, 16).

Antibodies directed against α_4 integrins and LFA-1 reduced the CH response (17, 18). Mice deficient in intracellular adhesion molecule 1 also have a reduced CH response (19). Similarly, antibodies against E-selectin and L-selectin diminished the recruitment of lymphocytes in delayed type hypersensitivity (20, 21). There are several reasons to think that P-selectin may also play a role in the CH response. P-selectin expression is regulated by various mediators present in the CH response, and it mediates adhesion of neutrophils, monocytes, and subsets of lymphocytes, all of which contribute to the inflammatory infiltrate in a CH reaction. P-selectin is partially responsible for rolling of $CD4^+$ T lymphocytes in vitro (22), and it is likely that lymphocytes, like other leukocytes, have to roll on the activated endothelium to infiltrate tissues. Since the rolling of leukocytes is practically absent in P-selectin–deficient mice (4), we decided to use the P-selectin–deficient mice to study the role of P-selectin in CH.

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Materials and Methods

Mice. 2- to 5-mo-old 129Sv C57BL wild-type or P-selectin-deficient females (4) were housed in the animal facilities at Massachusetts Institute of Technology (Cambridge, MA) or Tufts-New England Medical Center (Boston, MA).

Induction of CH to Oxazolone. On day 0, mice were painted on the inner surface of both hind legs with 25 μl oxazolone (Sigma Chemical Co., St. Louis, MO) (0.1 g/ml in 4:1 vol/vol acetone/olive oil); control animals received 25 μl of acetone/olive oil. The mice were challenged on day 5 with 5 μl of oxazolone on the inner side of the left pinna and vehicle on right pinna. 2 μCi [^{125}I]iododeoxyuridine (5.65 μCi/mg, Amersham Co., Arlington Heights, IL), in 0.1 ml PBS was injected into the tail vein 8 h after challenge (23), or 0.2 μCi ^{125}I-albumin (^{125}I-BSA, 1.59 μCi/mg, ICN Biomedicals, Inc., Costa Mesa, CA) (24) injection was given 16 h after challenge. 24 h after challenge, the mice were killed, and pinnae were cut off at the hairline and counted. This time point was chosen to reflect specific recruitment of inflammatory cells in response to the contact allergen (7). Ear thickness was measured in mice anesthetized with metafane (methoxyflurane; Pitman-Moore, Mundelein, IL), at 0 and 24 h after the challenge, using an engineer's micrometer.

Histology. Ears were cut in half longitudinally, and one-half was fixed in 10% formaldehyde, embedded in paraffin, and stained with hematoxylin and eosin (H&E). The other half was frozen with optimum cooling temperature compound (Miles, Inc., Diagnostic Division, Elkhart, IN), and sections were stained for CD4$^+$ lymphocytes with rat anti-mouse antibody L3T4 (American Type Culture Collection, Rockville, MD) and for macrophages with F4/80 antibody (American Type Culture Collection) using the Biotin-Streptavidin biotin system (Zymed Laboratories Inc., South San Francisco, CA) and Vectastain ABC kit (Vector Laboratories, Inc., Burlingame, CA). Serial sections from the same ears were stained with isotype-matched biotinylated rat IgG2b antibody as control (PharMingen, San Diego, CA). Neutrophils and mast cells were stained in paraffin-embedded sections for a specific esterase, using the naphthol AS-D chloroacetate esterase kit (Sigma Chemical Co.). Cells were counted throughout the entire section. All of the sections were examined independently by two investigators. The focal infiltrates in the epidermis were ellipsoid in shape. The length and width of each focal infiltrate was measured using a linear grid on a light microscope (BX40F; Olympus Corp., Lake Success, NY) in the H&E sections. The area of each focal infiltrate was calculated (area of an ellipsoid = $L/2 \times W/2 \times \pi$).

Results

To elicit contact hypersensitivity, wild-type and P-selectin-deficient mice were sensitized with oxazolone, and 5 d later they were challenged with the same substance on their left ear. The right ear was painted with vehicle. 24 h after the challenge, the ears of these mice were examined for mononuclear cell and neutrophil infiltration and for vascular permeability.

Infiltration of Radiolabeled Mononuclear Cells. Dividing monocytes and lymphocytes were labeled with [^{125}I]iododeoxyuridine 8 h after challenge (23), and infiltration of labeled cells at the site of the contact hypersensitivity reaction was determined 24 h after challenge by subtracting the counts in nonchallenged ears from those in challenged ears. The mutant animals had 53.8% lower infiltration ($P < 0.017$) of radiolabeled mononuclear cells (Fig. 1). Animals challenged with

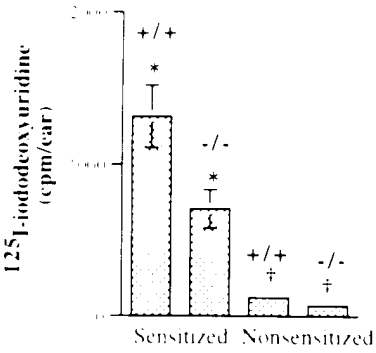

Figure 1. Infiltration of radiolabeled mononuclear cells. Oxazolone-sensitized wild-type (+/+) and P-selectin-deficient (−/−) mice were challenged 5 d later on their left ears. [^{125}I]Iododeoxyuridine was injected to label dividing mononuclear cells. 24 h after the challenge, the infiltration of radiolabeled cells into the ears of the sensitized and nonsensitized mice was determined; counts of the nonchallenged ears were subtracted from those of the challenged ears. Bars, mean ± SEM for each animal group. *$P < 0.017$, $n = 11$ for +/+ and $n = 13$ for −/−; †$P = 0.252$, $n = 4$ for +/+ and $n = 5$ for

oxazolone without prior sensitization showed much lower (<10%) infiltration of radiolabeled cells than did the sensitized animals (Fig. 1), and there was no statistical difference between wild-type and mutant animals.

Vascular Permeability. Vascular leak in delayed type hypersensitivity can be demonstrated by the local leakage of systemically injected radiolabeled albumin into the tissues (24). Since the vascular permeability in sensitized animals is reported to be maximum between 12 and 24 h (25), iodinated albumin was injected in the tail veins of mice 16 h after challenge, and both ears were counted 24 h after challenge. The leakage of radiolabeled albumin in mutant animals was 18.5% lower than in wild-type animals, but the difference was not statistically significant. Ear swelling was also measured using a micrometer before and 24 h after challenge. Change in thickness of ears before and after challenge was $2.55 \pm 0.017 \times 10^{-2}$ mm ($n = 17$) for the wild-type mice and $2.33 \pm 0.016 \times 10^{-2}$ mm ($n = 18$) for the mutants. The small difference in swelling (9%) was not statistically significant.

Histology of Ear Sections. The most notable feature of sections taken 24 h after challenge was the appearance of dense focal neutrophil infiltrates (confirmed by specific esterase staining) in the epidermis. These were less numerous and significantly smaller in the mutant than in wild-type mice (Fig. 2, *b*, *a*). Since the infiltrates were partially necrotic, it was not possible to count individual neutrophils. Therefore, the area of the focal collections was measured along the entire ear length and was 6.6 times smaller in the mutant mice (Fig. 3). The control nonchallenged ears had no focal infiltrates (Fig. 2, *c*, *d*). Nonsensitized mice had no or very few of these neutrophil clusters, ruling out the possibility that the neutrophil recruitment was an irritant response (Fig. 3). Necrosis of the epidermis was seen in the wild-type mice but was less prominent in the mutant mice. Neutrophil infiltration was also noted in the dermis (Fig. 2) but was more diffuse than in the epidermis. To determine neutrophil numbers in the

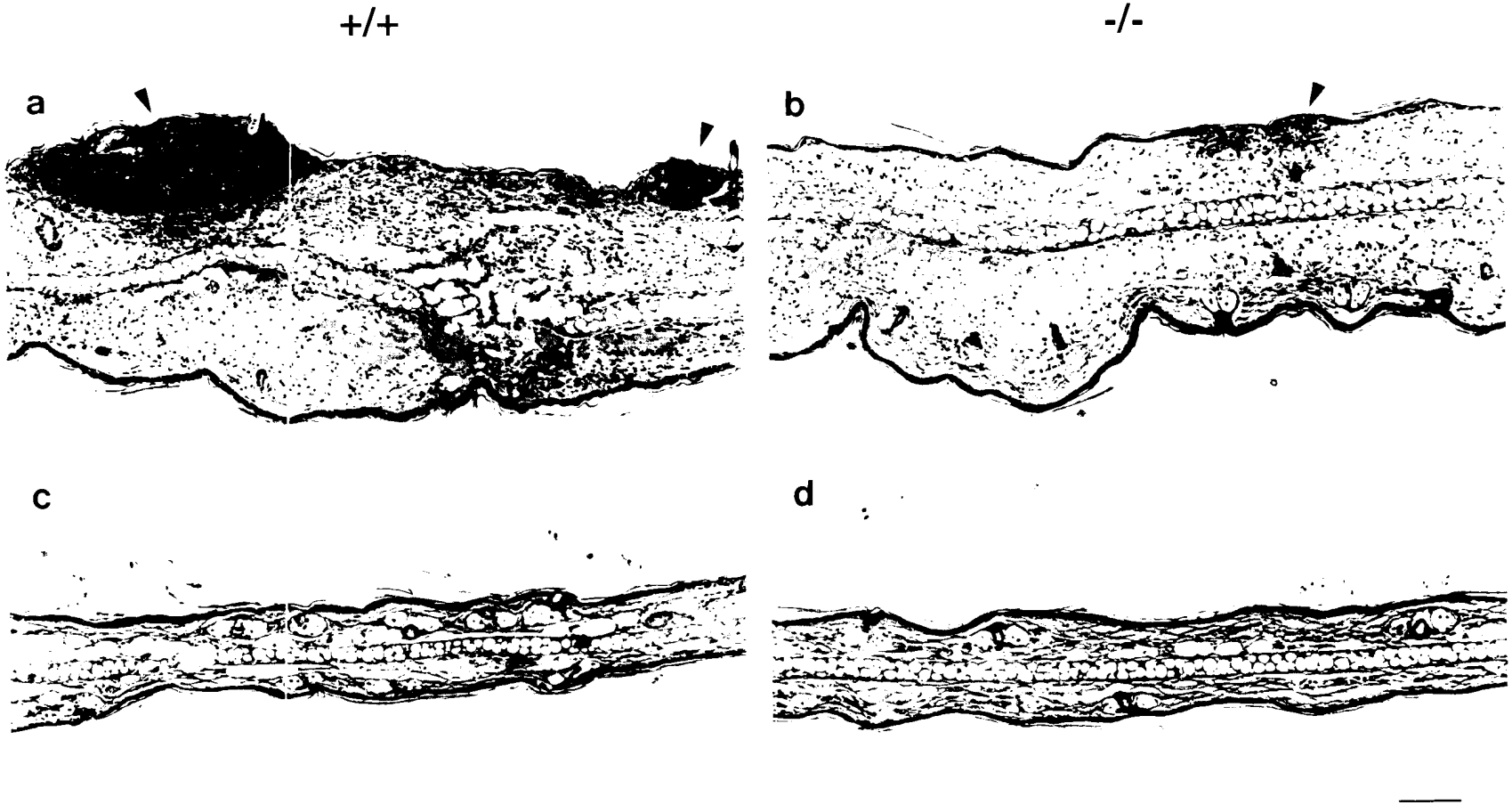

Figure 2. Histology of ear sections. Dense focal neutrophil infiltrates (arrows) were smaller in the challenged ears of the sensitized P-selectin deficient mice (-/-) (*b*) as compared with the wild-type mice (+/+) (*a*). Necrosis of the epidermis lining over these infiltrates was seen in the wild-type mice and to a lesser extent in the mutants. The control, nonchallenged ears of the sensitized wild-type (*c*) as well as the mutant mice (*d*) had no focal infiltrates. Bar, 100 μm.

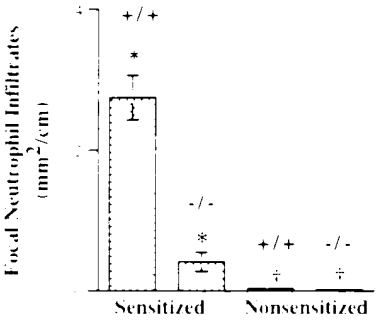

Figure 3. Focal neutrophil infiltrates in epidermis. The area of focal neutrophil infiltrates was measured along the entire ear length in H&E stained sections (see Fig. 2). Bars, mean ± SEM for each group of four animals. $*P < 0.001$; $\dagger P = 0.696$ (by Student's t test).

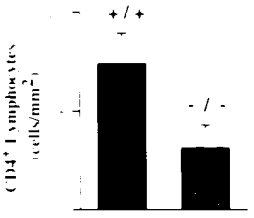

Figure 4. CD4$^+$ T lymphocyte infiltration of the dermis. Frozen sections of challenged ears from sensitized animals were stained using biotinylated antibody to CD4$^+$. Bar, mean + SEM ($n = 10$ for +/+, $n = 9$ for -/-). $P < 0.046$ (by Student's t test).

dermis, paraffin sections were stained with a specific esterase stain that identifies neutrophils and mast cells; the mast cells are larger and have a central nucleus. The challenged ears of the mutant mice had 47.7% fewer neutrophils in the dermis than did wild-type mice (Table 1). We also examined whether mast cell degranulation differed in the two genotypes. The specific esterase stain identifies the granules of mast cells (26); therefore, it is likely that degranulated mast cells would not stain. Numbers of mast cells stained were lower in both genotypes in the challenged ears than in control nonchallenged ears (Table 1). However, the wild-type mice had significantly more degranulation, as their numbers of positive mast cells were 1.6 times lower than in the mutants. Some degranulation was also observed in the challenged ears of the nonsensitized animals but was less compared with sensitized animals. There did not appear to be any degranulation in nonchallenged ears of the sensitized and the nonsensitized animals (Table 1).

The mononuclear cells measured by the incorporation of [^{125}I]iodode oxyuridine into dividing cells include both lymphocytes and monocytes (23). Monocytes macrophages were also stained with F4/80 antibody (27). Reduced numbers were seen in the mutant mice (data not shown), but the precise numbers could not be determined because of high nonspecific background. Since CD4$^+$ T lymphocytes are the effector cells in the contact hypersensitivity reaction, their influx into the inflammatory site was determined with an mAb to CD4$^+$. Parallel sections were stained with isotype-matched antibodies, and nonspecifically stained cells were counted and subtracted. The number of CD4$^+$ lymphocytes infiltrating the dermis of the challenged ears of sensitized P-selectin-deficient mice was 2.3 times lower ($P < 0.046$) than that in the sensitized wild-type mice (Fig. 4).

Discussion

Previously, our laboratory has demonstrated severe impairment in leukocyte endothelial interaction in P-selectin-deficient mice and delayed neutrophil recruitment in acute peritonitis (4). These results imply that recruitment of leukocytes may be altered in other inflammatory models, such as delayed hypersensitivity reaction, a condition of clinical relevance. Therefore, we studied a well-established murine model of CH in P-selectin–deficient mice. We observed that both mononuclear cells and neutrophils were diminished in the challenged ears of the sensitized P-selectin–deficient mice as compared to wild-type mice. Most interestingly, numbers of CD4$^+$ T lymphocytes in challenged ears of the P-selec-

Table 1. *Dermal Neutrophils and Mast Cells*

		Sensitized		Nonsensitized	
Genotype	Cell type	Challenged ear	Nonchallenged	Challenged ear	Nonchallenged
+ +	Neutrophils	622 ± 23*	12 ± 3	211 ± 125	8 ± 3
		(n = 4)	(n = 12)	(n = 4)	(n = 4)
	Neutrophils	296 ± 54*	6 ± 0.7	162 ± 32	9 ± 6
		(n = 4)	(n = 13)	(n = 2)	(n = 3)
+ +	Mast cells	30 ± 3‡	64 ± 7	40 ± 8	64 ± 13
		(n = 12)	(n = 10)	(n = 5)	(n = 5)
- -	Mast cells	49 ± 6‡	74 ± 7	69 ± 6	63 ± 8
		(n = 12)	(n = 12)	(n = 5)	(n = 4)

Paraffin-embedded sections of ears were stained with a specific esterase. The positive cells in all microscopic fields per tissue section were counted. Numbers represent cell count per square millimeter ± SEM. n indicates number of animals examined. *$P < 0.001$ by Student's t test; ‡$P < 0.005$ by Student's t test.

tin-deficient mice were half of that seen in the wild-type mice (Fig. 4). It is very likely that, like neutrophils, lymphocytes follow the multistep attachment process involving selectins and integrins (2). L- and E-selectin are used by lymphocytes for homing into lymph nodes and skin, respectively (28, 29). In another study, rolling of $CD4^+$ lymphocytes on TNF-α stimulated endothelium in vitro could be partially blocked by anti-P-selectin antibodies but not by E- or L-selectin antibodies (22). Although in vitro studies have shown that subsets of T lymphocytes bind P-selectin (3), its role in the actual migration to the sites of inflamed tissues has not been studied. In this study, we have demonstrated for the first time the role of P-selectin in tissue migration of $CD4^+$ lymphocytes. Since the migration of $CD4^+$ lymphocytes into tissues was not completely blocked in the P-selectin–deficient mice, it is likely that other adhesion molecules are being used.

A marked difference in the infiltration of neutrophils between the two genotypes was also observed in the present CH model. The epidermal focal infiltrates were sixfold higher and the dermal infiltrates were twofold higher in the wild-type mice. The greater severity of the epidermal reaction, in contrast to the dermal reaction, was probably due to the epicutaneous application of the sensitizing agent. In fact, the inflammation of the epidermis was seen primarily on the ventral side, where oxazolone was applied. The murine CH reaction, unlike the human prototype, has greater neutrophil infiltration (30). It is possible that there is a species difference in section up-regulation of distinct cytokines or chemokines in the CH reaction. It is known that cytokines and chemokines preferentially recruit different cell types (2).

We expected to see less edema in the P-selectin–deficient mice, since tissue infiltration of all inflammatory cells was reduced. However, only a marginal, nonsignificant decrease in edema was observed in the mutant animals. There are several possible reasons why the P-selectin–deficient mice did not show protection against edema. Although the mononuclear cells and neutrophils recruited in the mutant mice are fewer than in wild-type mice, it is possible that the numbers are sufficient to increase vascular permeability and that further recruitment in wild-type mice does not increase leakage any further. Alternatively, the two processes, leukocyte migration and leakage, could be independent of one another (31).

Since mast cell mediators, like histamine and serotonin, up-regulate P-selectin by releasing Weibel-Palade bodies (13, 14), we evaluated mast cell release in the CH reaction. In both genotypes, the nonchallenged ears had equivalent numbers of mast cells. The challenged ears of the wild-type mice had significantly fewer positive mast cells than did the nonchallenged ears. This was observed in the mutant mice as well, but to a significantly lesser extent, indicating that more mast cells degranulated in the wild-type mice. The more intense inflammatory response in the wild-type mice may be responsible for this observation. The actual role of mast cells in the development of contact hypersensitivity is not clear, because mast cell–deficient mice do not show in abnormal CH response (32). It is possible that the normal response in the mast cell–deficient mice may be due to an alternate source of serotonin from other cells like platelets (12).

P-selectin has been categorized as a molecule involved in acute inflammation because it is expressed on the surface within minutes after activation of endothelial cells (13). The results presented here clearly show an effect of the absence of P-selectin on a long-term inflammatory response. It is conceivable that this late effect reflects an earlier defect. For instance, P-selectin might play some role in the sensitization phase. Alternately, it is possible that the general reduction in cellular recruitment we observed 24 h after challenge was due, at least in part, to defective infiltration in the first hours of the response. Reduced numbers of recruited cells in the first hours, due to lack of P-selectin expression, might release lower amounts of cytokines and chemotactic proteins and thus result in lesser endothelial activation and lower additional cellular recruitment. It is also possible that, in the absence of P-selectin, the mononuclear cells are not fully activated, as P-selectin was recently shown to regulate cytokine secretion by human monocytes (33). On the other hand, since P-selectin is known to be transcriptionally regulated by cytokines (34), it is very likely to contribute directly to the late recruitment. Moreover, we have shown that after surface expression in endothelial cells, P-selectin is endocytosed, and a portion of the molecules travels into nascent storage granules and is therefore available for reuse (35). CH may represent a model that highlights P-selectin's role in long-term leukocyte recruitment. Here, for the first time, we have also demonstrated the crucial role that P-selectin plays in recruitment of $CD4^+$ T lymphocytes into inflammatory lesions.

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